

# Commensal bacteria can enter colonic epithelial cells and induce proinflammatory cytokine secretion: a possible pathogenic mechanism of ulcerative colitis

Toshifumi Ohkusa,<sup>1</sup> Tsutomu Yoshida,<sup>2</sup> Nobuhiro Sato,<sup>3</sup>  
Sumio Watanabe,<sup>3</sup> Hisao Tajiri<sup>1</sup> and Isao Okayasu<sup>2</sup>

Correspondence  
Toshifumi Ohkusa  
ohkusa@jikei.ac.jp

<sup>1</sup>Department of Gastroenterology and Hepatology, The Jikei University School of Medicine, Kashiwa, Chiba 277-8567, Japan

<sup>2</sup>Department of Pathology, Kitasato University School of Medicine, Sagamihara, Kanagawa 228-8555, Japan

<sup>3</sup>Department of Gastroenterology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

Interleukin 2 (IL-2)- and IL-10-knockout mice develop spontaneous colitis under conventional but not germ-free conditions, suggesting that commensal bacteria play an important role in the pathogenesis of colitis. However, interactions between commensal bacteria and colonic epithelial cells have not been fully investigated. We therefore assessed the ability of various commensal bacteria and probiotics to adhere to and invade colonic epithelial cells. Effects of the bacteria on production of proinflammatory cytokines were also measured. Commensal bacteria, including mucosal organisms isolated from ulcerative colitis (UC) patients, such as *Fusobacterium varium*, reported as a possible pathogen in UC, *Bacteroides vulgatus*, *Escherichia coli* and *Clostridium clostridioforme*, as well as their type strains and probiotics, were assessed for their ability to adhere to and invade colonic epithelial cells using two cell lines, SW-480 and HT-29. Our experiments employed co-incubation, a combination of scanning and transmission electron microscopy and recovery of bacteria from infected-cell lysates. *F. varium* and several other commensal bacteria, but not probiotics, adhered to colonic epithelial cells and invaded their cytoplasm. ELISA and real-time PCR revealed that the host cells, particularly those invaded by *F. varium*, showed significant increases in IL-8 and TNF- $\alpha$  concentrations in supernatants, with elevation of IL-8, TNF- $\alpha$ , MCP-1 and IL-6 mRNAs. Furthermore, IL-8 and TNF- $\alpha$  expression and nuclear phosphorylated NF- $\kappa$ B p65 expression could be immunohistochemically confirmed in inflamed epithelium with cryptitis or crypt abscess in UC patients. Certain commensal bacteria can invade colonic epithelial cells, activating early intracellular signalling systems to trigger host inflammatory reactions.

Received 12 August 2008  
Accepted 5 December 2008

## INTRODUCTION

The aetiology of ulcerative colitis (UC) is still not understood in detail, although several possible mechanisms have been proposed. Since IL-2- and IL-10-knockout mice develop spontaneous colitis under routine but not germ-free conditions, commensal bacteria may play an important role in the pathogenesis of colitis (Sadlack *et al.*, 1993; Sellon *et al.*, 1998). There is growing evidence that numbers of mucosa-associated bacteria, especially anaerobic *Bacteroides* species and aerobic members of the *Enterobacteriaceae*

(*Escherichia coli*), are dramatically increased in inflammatory bowel diseases, while numbers of lactic acid bacilli are decreased (Swidsinski *et al.*, 2002; Guarner *et al.*, 2002; Mylonaki *et al.*, 2005). Consistent with these observations, we have found significantly increased detection rates of *Fusobacterium varium* in actively inflamed colonic mucosa of patients with UC and *F. varium*-induced specific antibodies in serum (Ohkusa *et al.*, 2002). Furthermore, an enema with butyric acid produced by *F. varium* can induce apoptosis in murine colonic epithelium, resulting in crypt abscess formation and UC-like lesions, as well as induce apoptosis in an *in vitro* cell culture (Ohkusa *et al.*, 2003; Yoshida *et al.*, 2006).

Abbreviations: IL, interleukin; SEM, scanning electron microscopy; TEM, transmission electron microscopy; UC, ulcerative colitis.

Recent studies have revealed that intestinal epithelial cells, the first host cells to come in contact with enteric pathogens, secrete a chemotactic mediator in response to pathogenic bacterial attachment or entry. It is well known that enteropathogenic bacteria invade these epithelial cells and stimulate IL-8 production (Eckmann *et al.*, 1993a; Aihara *et al.*, 1997; Vitiello *et al.*, 2004; Singer & Sansonetti, 2004). Evidence has been presented that intestinal epithelial cells have the capacity to express and secrete several cytokines constitutively (Eckmann *et al.*, 1993b; Jung *et al.*, 1995; Weinstein *et al.*, 1997; Kuhara *et al.*, 2000; Sartor, 2004) when stimulated by pathogenic bacteria. Studies on critical constituents of the innate immune response, such as Toll-like receptors and nucleotide binding oligomerization domain isoforms, also suggest that bacterial infections may contribute to intestinal inflammation (Hoshino *et al.*, 1999; Ohkusa *et al.*, 2004). However, it is currently unclear whether epithelial cells secrete cytokines in response to nonpathogenic commensal bacteria or probiotics. In the present study, we found that the production of cytokines by colonic epithelial cells appears to be an important primary event in host defence, by analysing an *in vitro* model system in which commensal bacteria attach to or penetrate epithelial cells. Furthermore, we defined secretion of inflammatory cytokines and mRNA expression employing co-culture experiments. Moreover, we tested immunohistochemical expression of inflammatory cytokines and activated NF- $\kappa$ B in the inflamed epithelium in patients with UC.

## METHODS

**Bacterial strains and attachment and invasion assays.** As listed in Table 1, we used four commensal bacterial strains, *F. varium*, *E. coli*, *Bacteroides vulgatus* and *Clostridium clostridioforme*, isolated from patients with UC (Ohkusa *et al.*, 2002); four type strains, *E. coli* JCM 1649<sup>T</sup>, *B. vulgatus* JCM 5826<sup>T</sup>, *C. clostridioforme* JCM 1291<sup>T</sup> and

*F. varium* ATCC 8501<sup>T</sup> (Burke & Axon, 1988; Matsuda *et al.*, 2000); and two probiotics, *Lactobacillus johnsonii* (La1 strain; Nestec collection, Lausanne, Switzerland) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LB-021001; Meiji Dairies). Bacteria were harvested for attachment and invasion assays after incubation on GAM agar plates (Nissui Chemicals) under aerobic conditions with 5% CO<sub>2</sub> or under anaerobic conditions (anaerobic chamber) with 10% CO<sub>2</sub>, 5% H<sub>2</sub> and 85% N<sub>2</sub> for 2–4 days at 37 °C. They were then suspended at 1 × 10<sup>8</sup> cells ml<sup>-1</sup> in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Cellgro Mediatech) and Ham's F-12 medium (Cellgro Mediatech).

Bacterial attachment and invasion assays were conducted according to the methods of Han & Miller (1997) with modifications for intestinal bacteria. We chose experiments on SW-480 and HT-29 cells (SW-480, ATCC CCL228; HT-29, ATCC HTB-38), commonly studied human intestinal epithelial cell lines, because they exhibit a highly LPS-responsive phenotype and surface expression of the TLR4 protein (Suzuki *et al.*, 2003). Human colonic epithelial cells were grown to near confluence (3.3–4.0 × 10<sup>5</sup> cells per well) in 6-well culture plates before infection with the bacteria in a 1:1 mixture of DMEM and Ham's F-12 medium containing 10% fetal bovine serum. Bacteria were added at a m.o.i. of 1000 (c.f.u.) and incubated under 5% CO<sub>2</sub> at 37 °C. For the attachment assay, supernatants were collected and stored at -80 °C for ELISAs after co-incubation with bacteria for 1 h. Then, after washing the SW-480 monolayers four times, total cell-associated bacteria were counted on GAM agar plates after cell lysis with water for the attachment assay. For the invasion assay, the monolayers were washed twice with sterile PBS after co-incubation with bacteria for 3 h and incubated in fresh medium containing imipenem (50 µg ml<sup>-1</sup>) for an additional 1 h to kill extracellular bacteria. After washing twice, the monolayers were further incubated for 1 h in fresh medium. The supernatant was then collected and stored at -80 °C for ELISA. The monolayers were rinsed again with PBS and lysed with sterile water. Internalized bacteria were counted on agar plates (GAM agar plates for *B. vulgatus*, *F. varium*, *Lactobacillus johnsonii* and *L. delbrueckii* subsp. *bulgaricus*, and trypticase soy agar plates for *E. coli*). Preliminary experiments confirmed that bacterial viability was unaffected by the water treatment during cell lysis. The levels of attachment and invasion were expressed as the percentage of bacteria retrieved following cell lysis relative to the total number of bacteria initially added.

**Table 1.** Bacterial attachment to and invasion of SW-480 cells

The assays were carried out as described in the text. Shown here are the mean results of assays with standard deviations. Each experiment was carried out in duplicate.

Strain	Attachment (%)*	SEM	Invasion (%)*	TEM
<i>F. varium</i> 113	0.034 ± 0.008†	2+	0.014 ± 0.004†	2+
<i>F. varium</i> ATCC 8501 <sup>T</sup>	0.029 ± 0.019†	2+	0.008 ± 0.003†	2+
<i>E. coli</i> R-1	0.070 ± 0.032†	+	0.0003 ± 0.00001	–
<i>E. coli</i> JCM 1649 <sup>T</sup>	0.030 ± 0.014†	+	0.0002 ± 0.0001	+
<i>B. vulgatus</i> 90	0.018 ± 0.004†	+	0.004 ± 0.001	+
<i>B. vulgatus</i> JCM 5826 <sup>T</sup>	0.013 ± 0.004†	+	0.002 ± 0.001	+
<i>C. clostridioforme</i> 94	0.003 ± 0.002	+	0.003 ± 0.001	+
<i>C. clostridioforme</i> JCM 1291 <sup>T</sup>	0.002 ± 0.001	+	0	–
<i>L. johnsonii</i> La1	0.023 ± 0.023	+	0.0004 ± 0.0001	–
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> LB-021001	0.008 ± 0.004	–	0.00006 ± 0.00002	–

\*The attachment and invasion levels were expressed as the percentage of bacteria retrieved following cell lysis relative to the total number of bacteria initially added.

†*P* < 0.05 by ANOVA when compared with *L. delbrueckii* subsp. *bulgaricus* LB-021001.

**Scanning and transmission electron microscopy (SEM and TEM) analysis.** For attachment, the SW-480 monolayers were replaced with *tert*-butyl alcohol, freeze-dried, coated with an osmium plasma coater, and analysed by SEM. For invasion, monolayers were fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer and osmium tetroxide, followed by dehydration and epon embedding. Ultrathin sections were stained with uranyl acetate and lead citrate and analysed by TEM. In experiments in which adherence and invasion levels were determined, 30 observation fields obtained at a magnification of  $\times 5000$  were randomly chosen and the number of attached or invading bacteria was semiquantified as: +, bacteria found in 1–5/30 fields; and 2+, bacteria found in  $>6/30$  fields.

**Detection of secreted cytokines in supernatants.** To determine the amounts of IL-8, IL-6, MCP-1, TNF- $\alpha$  and IL-18 secreted by SW-480 or HT-29 cells co-incubated with bacteria for 1 h or 1–3 h, ELISAs were conducted using commercial assay kits (IL-8 EASIA, BioSource Europe; QuantiGlo Human IL-6 Immunoassay, R&D Systems; Quantikine Human MCP-1 Immunoassay, R&D Systems; QuantiGlo Human TNF- $\alpha$  Immunoassay, R&D Systems; Human IL-18 ELISA kit, Medical & Biological Lab.), according to the manufacturers' protocols. All experiments were performed in triplicate and repeated at least three times. Results are shown as mean  $\pm$  standard deviation (SD).

**Semiquantitative real-time PCR.** Real-time (TaqMan) RT-PCR for mRNA was conducted as described by Mitchell *et al.* (2003). Briefly, RNA was prepared, as described above, from monolayered SW-480 or HT-29 cells infected with the test bacteria during a 1 h or 1–3 h incubation. Total RNA was isolated using an RNeasy Mini kit (Qiagen), and DNase treatment was performed using an RNase-Free DNase Set (Qiagen). Reverse transcription with up to 4  $\mu$ g total RNA was carried out in a total volume of 20  $\mu$ l containing 250 pmol random primer, 100 U SuperScript II RNase H-Reverse Transcriptase (Invitrogen) in 50 mM Tris/HCl (pH 8.3), 40 mM KCl, 6 mM MgCl<sub>2</sub>, 1 mM DTT and 10 mM dNTPs, following the manufacturer's instructions. Initially, a total RNA solution mixed with random primer was heated at 70 °C for 10 min and immediately chilled on ice, and the other reagents were then added. First-strand cDNAs were obtained after 50 min at 42 °C and 5 min at 98 °C.

Primers and TaqMan probes for target genes (IL-8, IL-6, MCP-1, TNF- $\alpha$  and IL-18) were obtained from TaqMan Gene Expression Assays and an internal reference gene ( $\beta$ -actin) from Pre-Developed TaqMan Assay Reagents (Applied Biosystems). Each of the TaqMan probes was labelled with a reporter dye (FAM or VIC) situated at the 5'-end of the oligonucleotide and a quencher dye (MGB) located at the 3'-end. The TaqMan Gene Expression Assay numbers are Hs00174131\_m1 for IL-6, Hs00174103\_m1 for IL-8, Hs00155517\_m1 for IL-18, Hs00234140\_m1 for MCP-1, Hs00174128\_m1 for TNF- $\alpha$  and 4326315E for  $\beta$ -actin. Quantification of target genes (IL-8, IL-6, MCP-1, TNF- $\alpha$  and IL-18) and the reference gene ( $\beta$ -actin) was performed in 96-well plates with ABI PRISM 7700 Sequence Detection System (ABI) data collection, and analyses were performed using the machine software. The PCR was carried out in a final volume of 25  $\mu$ l containing cDNA equivalent to 10–100 ng total RNA, 12.5  $\mu$ l 2 $\times$  TaqMan Universal PCR Master Mix and 1.25  $\mu$ l 20 $\times$  TaqMan Expression Assay reagent. Thermal cycler conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The comparative CT method of data analysis was used to analyse the data. CT is the PCR cycle at which an increase in reporter fluorescence above the baseline level is first detected. The target gene CT and internal reference gene CT were calculated for each sample.  $\Delta$ CT is the difference in CT between the target and reference genes;  $\Delta\Delta$ CT is the difference in  $\Delta$ CT for the sample and the calibrator sample. The amount of target gene expression, normalized to an internal reference and

relative to the calibrator sample, is given by  $2^{-\Delta\Delta\text{CT}}$ . Each sample was analysed in triplicate and each assay was repeated three times. Results are shown as mean  $\pm$  standard error (SE).

**Immunohistochemistry for clinical samples.** For immunohistochemistry of cytokines and NF- $\kappa$ B in rectal mucosae of patients with UC, surgically removed rectums were obtained from patients with colorectal cancers (10 cases), inactive UC (12 cases) and active UC (7 cases). The active and inactive phases of UC were defined using Seo's complex integrated disease activity indices for clinical UC activity (Seo *et al.*, 1992). Immunohistochemical staining of new sets of serial sections (4  $\mu$ m in thickness) from formalin-fixed, paraffin-embedded blocks was achieved using an EnVision+ kit (Dako), as in our previous study (Mikami *et al.*, 2003; Mitsuhashi *et al.*, 2005). The primary antibodies and their working dilutions were as follows: mouse monoclonal anti-TNF- $\alpha$  (clone 28401,  $\times 50$ ; R&D Systems), rabbit anti-human IL-8 ( $\times 100$ ; MONOSAN), mouse anti-NF- $\kappa$ B subunit p65 (clone 20,  $\times 500$ ; BD Transduction Laboratory) and rabbit anti-phosphor-NF- $\kappa$ B p65 (Ser276) antibodies ( $\times 100$ ; Cell Signaling Technology). Briefly, after a 15 min microwave pretreatment in citrate buffer (pH 6.0, 0.01 M) for retrieval of the four antigens, sections were incubated with the primary antibodies for 1 h at room temperature. Nuclei were counterstained with methyl-green solution to facilitate histopathological assessment. Crypts bearing at least two positive epithelia were considered to be positive. Two full-length crypts adjacent to the muscularis mucosa were randomly selected and observed in normal mucosa more than 2 cm from the tumour in colorectal cancer cases and mucosa in inactive UC, with neither cryptitis nor crypt abscess. Crypts with cryptitis or crypt abscess were randomly selected from seven active UC cases and observed.

**Ethics.** This work using pathological samples was approved by our Kitasato University Medical School and University Hospital Ethics Committee.

**Statistical analysis.** Data are presented as means  $\pm$  SE or SD and range. Statistical analysis was performed using repeated measures ANOVA and least significant difference post hoc tests for multiple comparisons. The  $\chi^2$  test was applied to compare the frequency of immunopositive crypts. Differences were considered statistically significant at  $P < 0.05$ .

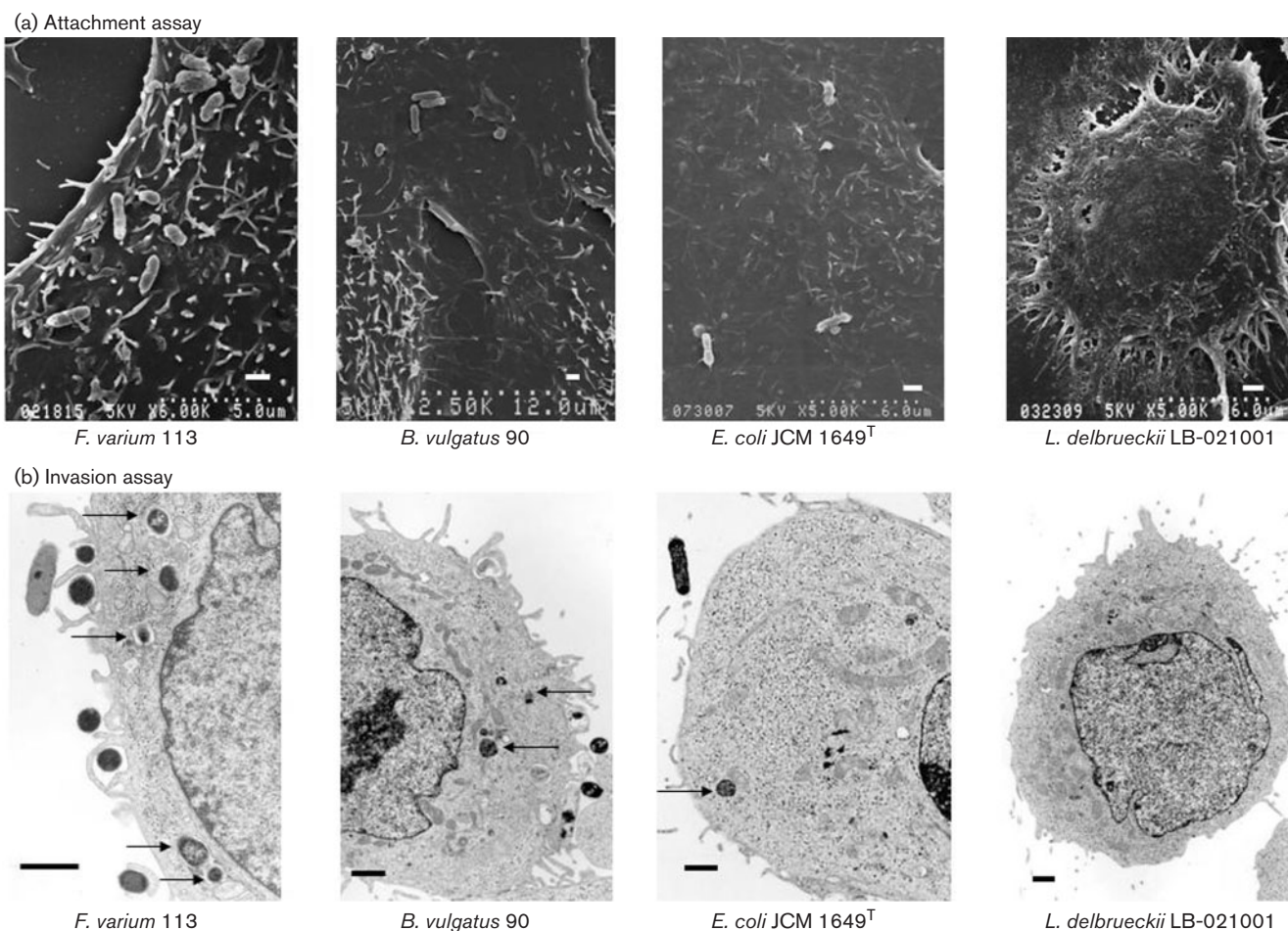
## RESULTS AND DISCUSSION

### Bacterial attachment and invasion

To measure bacterial attachment to and invasion of SW-480 cells, SEM and TEM as well as counting on agar plates (recovery of bacteria) after cell lysis were employed (Fig. 1). Counting on agar plates after cell lysis showed that the attachment levels of *F. varium* 113 and ATCC 8501<sup>T</sup>, *E. coli* R-1 and JCM 1649<sup>T</sup>, *B. vulgatus* 90 and JCM 5826<sup>T</sup>, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, and *L. johnsonii* La were approximately 2–10-fold higher than that of *L. delbrueckii* subsp. *bulgaricus* LB-021001 by the co-incubation method. SEM revealed *F. varium* 113 and ATCC 8501<sup>T</sup> to be more adhesive than other bacterial strains to these cells (Table 1; Fig. 1a).

In the invasion assay, TEM revealed that *E. coli* R-1, *C. clostridioforme* JCM 1291<sup>T</sup> and the two probiotics did not invade SW-480 cells. *E. coli* JCM 1649<sup>T</sup>, *B. vulgatus* 90 and





**Fig. 1.** Adherence and invasion of commensal bacteria and probiotics in SW-480 cells (SEM and TEM). (a) The scanning electron micrographs show attachment of bacteria, with the exception of *L. delbrueckii* subsp. *bulgaricus* LB-021001, to SW-480 cells. *F. varium* 113 was more adhesive than the other bacterial strains tested. Bar, 1  $\mu$ m. (b) Commensal bacteria, *F. varium* 113 and *B. vulgatus* 90, isolated from patients with UC, and *E. coli* JCM 1649<sup>T</sup> adhere to SW-480 cells and invade their cytoplasm (arrows), while the probiotic *L. delbrueckii* subsp. *bulgaricus* does not enter the cells (TEM photograph; bar, 1  $\mu$ m).

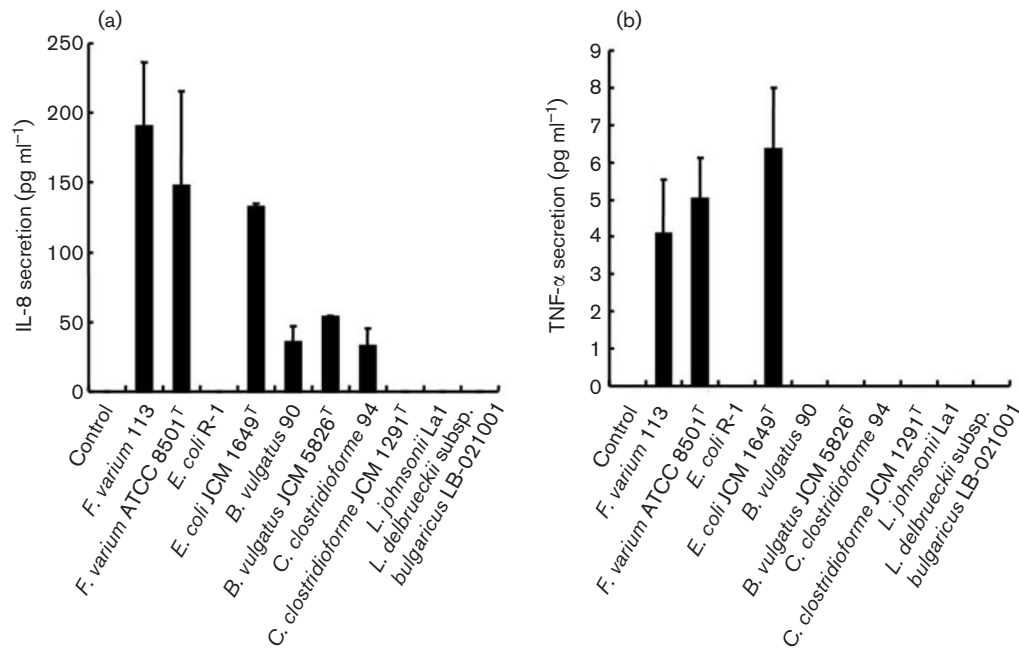
JCM 5826<sup>T</sup>, and *C. clostridioforme* 94 showed weak invasion. *F. varium* 113 and ATCC 8501<sup>T</sup> strains showed much greater invasion of SW-480 cells, both by counts on agar plates and TEM (Fig. 1b). The present study revealed using the co-incubation method that *F. varium* and several other commensal bacteria, but not probiotics, adhered to colonic epithelial cells and invaded their cytoplasm.

### Cytokine production by bacterial attachment and invasion

Following the above described results, to determine the production of cytokines upon infection with commensal bacteria and probiotics, the levels of IL-8, IL-6, MCP-1, TNF- $\alpha$  and IL-18 released by SW-480 and HT-29 in the bacterial attachment assay were measured in culture supernatants. However, concentrations of the cytokines IL-8 (<16 pg ml<sup>-1</sup>), IL-6 (<1.5 pg ml<sup>-1</sup>), MCP-1

(<31.2 pg ml<sup>-1</sup>), TNF- $\alpha$  (<0.6 pg ml<sup>-1</sup>) and IL-18 (<15.6 pg ml<sup>-1</sup>) were all below their detection limits. For the invasion assay, significantly increased IL-8 production by SW-480 (Fig. 2a) and HT-29 (Fig. 3a) was induced by *F. varium* 113 (mean  $\pm$  SD; 95% confidence interval, not shown) (SW-480, 190.5  $\pm$  18.8 pg ml<sup>-1</sup>; HT-29, 188.8  $\pm$  15.0 pg ml<sup>-1</sup>, respectively), *F. varium* ATCC 8501<sup>T</sup> (135.3  $\pm$  30.5; 147.6  $\pm$  68.3), *E. coli* JCM 1649<sup>T</sup> (132.5  $\pm$  6.1; 125.3  $\pm$  21.7), *B. vulgatus* JCM 5826<sup>T</sup> (53.2  $\pm$  1.37; 50.7  $\pm$  3.24), *B. vulgatus* 90 (35.1  $\pm$  11.7; 30.3  $\pm$  7.8) and *C. clostridioforme* 94 (32.8  $\pm$  12.7; 33.3  $\pm$  9.5). Noninvasive bacteria, i.e. *E. coli* R-1, *C. clostridioforme* JCM 1291<sup>T</sup>, *L. johnsonii* La1 and *L. delbrueckii* subsp. *bulgaricus* LB-021001, failed to stimulate IL-8 secretion.

In the invasion assay, TNF- $\alpha$  production by SW-480 (Fig. 2b) and HT-29 cells (Fig. 3b) was clearly induced by *E. coli* JCM 1649<sup>T</sup> (mean  $\pm$  SD; 95% confidence interval, not shown) (SW-480, 5.1  $\pm$  0.6 pg ml<sup>-1</sup>; HT-29,



**Fig. 2.** Production of cytokines upon infection with commensal bacteria and probiotics in SW-480 cells. (a) IL-8. In the invasion assay, *F. varium* 113 stimulated the greatest IL-8 production, followed by *F. varium* ATCC 8501<sup>T</sup>, *E. coli* JCM 1649<sup>T</sup>, *B. vulgatus* JCM 5826<sup>T</sup>, *B. vulgatus* 90 and *C. clostridioforme* 94. Since the IL-8 levels at the 95 % confidence interval for these bacteria were above those of the controls (<16 pg ml<sup>-1</sup>), these bacteria significantly stimulated IL-8 production by SW-480. The IL-8 levels with *F. varium* 113, ATCC 8501<sup>T</sup> and *E. coli* JCM 1649<sup>T</sup> were significantly higher than those with *B. vulgatus* 90 and *C. clostridioforme* 94 ( $P < 0.01$ ). (b) TNF- $\alpha$ . In the invasion assay, commensal bacterial strains, i.e. *E. coli* JCM 1649<sup>T</sup> and *F. varium* 113 and ATCC 8501<sup>T</sup>, induced TNF- $\alpha$  production by SW-480 cells. *E. coli* JCM 1649<sup>T</sup> stimulated the greatest TNF- $\alpha$  production, followed by *F. varium* ATCC 8501<sup>T</sup> and *F. varium* 113. Since the TNF- $\alpha$  levels at the 95 % confidence interval for the bacteria were above those of the controls, these bacteria significantly stimulated TNF- $\alpha$  production by SW-480 cells.

$6.4 \pm 1.6$  pg ml<sup>-1</sup>), *F. varium* ATCC 8501<sup>T</sup> ( $5.1 \pm 0.7$ ;  $6.6 \pm 1.3$ ) and *F. varium* 113 ( $4.2 \pm 0.7$ ;  $6.9 \pm 1.1$ ). However, IL-6, MCP-1 and IL-18 were below their detection limits for all of the bacteria tested.

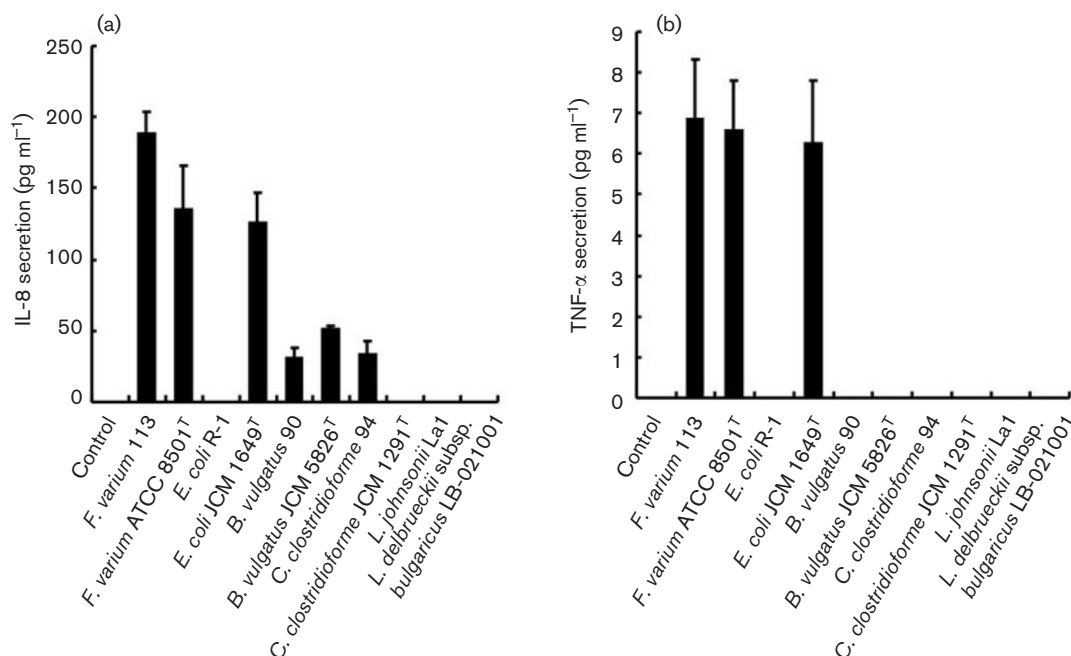
### mRNA expression of cytokines by bacterial attachment and invasion

Following production of cytokines, real-time RT-PCR was conducted to measure the induction of cytokine mRNAs upon infection with commensal bacteria and probiotics. The bacteria tested, with the exception of *C. clostridioforme* JCM 1291<sup>T</sup> and *L. johnsonii* La1 strains in the attachment assay, and commensal bacteria, with the exception of *C. clostridioforme* JCM 1291<sup>T</sup> and probiotics in the invasion assay with SW-480 (Fig. 4a), and with the exception of *L. johnsonii* La1 strains in the attachment assay with HT-29 cells (Fig. 5a), markedly stimulated IL-8 mRNA expression. In the invasion assays, IL-8 mRNA expression was stimulated by *F. varium* ATCC 8501<sup>T</sup> (relative fold increase, mean  $\pm$  SE; 95 % confidence intervals not shown) (SW-480,  $447.9 \pm 13.4$ ; HT-29,  $130.3 \pm 7.2$ , respectively), *F. varium* 113 ( $115.1 \pm 2.8$ ;  $152.7 \pm 8.3$ ) and *E. coli* JCM 1649<sup>T</sup> ( $102.9 \pm 0.5$ ;  $119.4 \pm 4.2$ ). Since the 95 % confidence

intervals for IL-8 mRNA levels in cells infected with all bacterial strains were above those of the controls, these bacteria were confirmed to stimulate IL-8 mRNA expression in SW-480 and HT-29 cells. However, the stimulated IL-8 mRNA expression levels varied with the bacterial strains, and noninvasive bacteria, i.e. *E. coli* R-1, *C. clostridioforme* JCM 1291<sup>T</sup>, *L. johnsonii* La1 and *L. delbrueckii* subsp. *bulgaricus* LB-021001, slightly stimulated IL-8 mRNA expression.

The bacteria tested, with the exception of *C. clostridioforme* JCM 1291<sup>T</sup>, *L. johnsonii* La1 and/or *L. delbrueckii* subsp. *bulgaricus* LB-021001 strains in the attachment assay, and *C. clostridioforme* JCM 1291<sup>T</sup> in the invasion assay, stimulated TNF- $\alpha$  mRNA expression by SW-480 and HT-29 cells (Figs 4b, 5b). In the invasion assays, TNF- $\alpha$  mRNA expression was stimulated by *F. varium* ATCC 8501<sup>T</sup> (SW-480,  $115.2 \pm 4.2$ ; HT-29,  $29.7 \pm 5.9$ ), *F. varium* 113 ( $34.8 \pm 1.2$ ;  $30.6 \pm 1.3$ ), *E. coli* JCM 1649<sup>T</sup> ( $36.6 \pm 2.5$ ;  $26.8 \pm 2.7$ ) and *B. vulgatus* JCM 5826<sup>T</sup> ( $14.1 \pm 0.9$ ;  $13.3 \pm 0.7$ ).

*F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, and *B. vulgatus* JCM 5826<sup>T</sup> strains in the attachment assay, as well as *B. vulgatus*



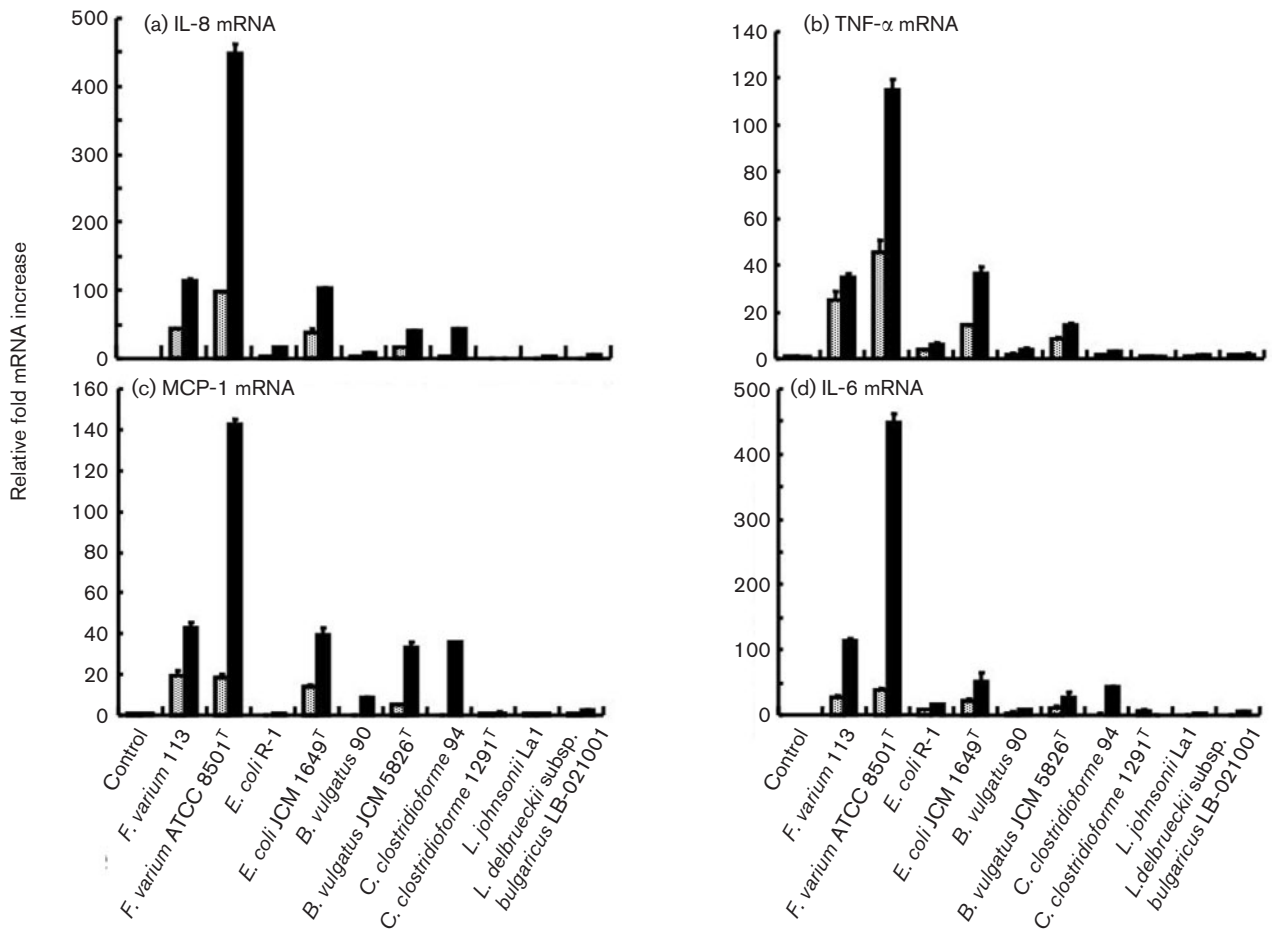
**Fig. 3.** Production of cytokines upon infection with commensal bacteria and probiotics in HT-29 cells. (a) IL-8. In the invasion assay, *F. varium* 113 stimulated the greatest IL-8 production, followed by *F. varium* ATCC 8501<sup>T</sup>, *E. coli* JCM 1649<sup>T</sup>, *B. vulgatus* JCM 5826<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* 90. Since the IL-8 levels at the 95% confidence interval for these bacteria were above those of the controls (<16 pg ml<sup>-1</sup>), these bacteria significantly stimulated IL-8 production by HT-29 cells. The IL-8 levels with *F. varium* 113 and ATCC 8501<sup>T</sup> and *E. coli* JCM 1649<sup>T</sup> were significantly higher than those with *B. vulgatus* 90 and *C. clostridioforme* 94 ( $P < 0.0001$ ). (b) TNF- $\alpha$ . In the invasion assay, commensal bacterial strains, i.e. *F. varium* 113, ATCC 8501<sup>T</sup> and *E. coli* JCM 1649<sup>T</sup>, induced TNF- $\alpha$  production by HT-29 cells. *F. varium* 113 stimulated the greatest TNF- $\alpha$  production, followed by *F. varium* ATCC 8501<sup>T</sup> and *E. coli* JCM 1649<sup>T</sup>. Since the TNF- $\alpha$  levels at the 95% confidence interval for the bacteria were above those of the controls, these bacteria significantly stimulated TNF- $\alpha$  production by HT-29 cells.

90, *C. clostridioforme* 94 and *E. coli* R-1 tested in the invasion assay significantly stimulated MCP-1 mRNA expression in SW-480 and HT-29 cells (Figs 4c, 5c). In the invasion assay, *F. varium* 113 (SW-480,  $42.6 \pm 3.1$ ; HT-29,  $41.1 \pm 2.3$ ), *F. varium* ATCC 8501<sup>T</sup> ( $142.1 \pm 3.1$ ;  $40.6 \pm 2.7$ ), *E. coli* JCM 1649<sup>T</sup> ( $39.6 \pm 3.1$ ;  $38.0 \pm 2.3$ ), *B. vulgatus* JCM 5826<sup>T</sup> ( $33.7 \pm 2.6$ ;  $30.4 \pm 1.5$ ) and *C. clostridioforme* 94 ( $35.6 \pm 0.6$ ;  $32.6 \pm 1.2$ ) strains stimulated marked MCP-1 mRNA expression.

*F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and R-1, and *B. vulgatus* JCM 5826<sup>T</sup> strains in the attachment assay, as well as the bacteria tested with the exception of *L. johnsonii* La1 in the invasion assay, significantly stimulated IL-6 mRNA expression in SW-480 and HT-29 cells (Figs 4d, 5d). In the invasion assays, IL-6 mRNA expression was stimulated higher by *F. varium* ATCC 8501<sup>T</sup> (SW-480,  $447.9 \pm 13.4$ ; HT-29,  $145.4 \pm 11.5$ ), *F. varium* 113 ( $115.1 \pm 2.8$ ;  $113.8 \pm 2.2$ ), *E. coli* JCM 1649<sup>T</sup> ( $52.1 \pm 11.9$ ;  $50.4 \pm 4.3$ ), *C. clostridioforme* 94 ( $42.4 \pm 1.0$ ;  $40.9 \pm 1.4$ ) and *B. vulgatus* JCM 5826<sup>T</sup> ( $27.0 \pm 7.4$ ;  $26.5 \pm 4.3$ ). IL-18 mRNA expression was not detected in either bacterially stimulated or unstimulated SW-480 cells. This is in line

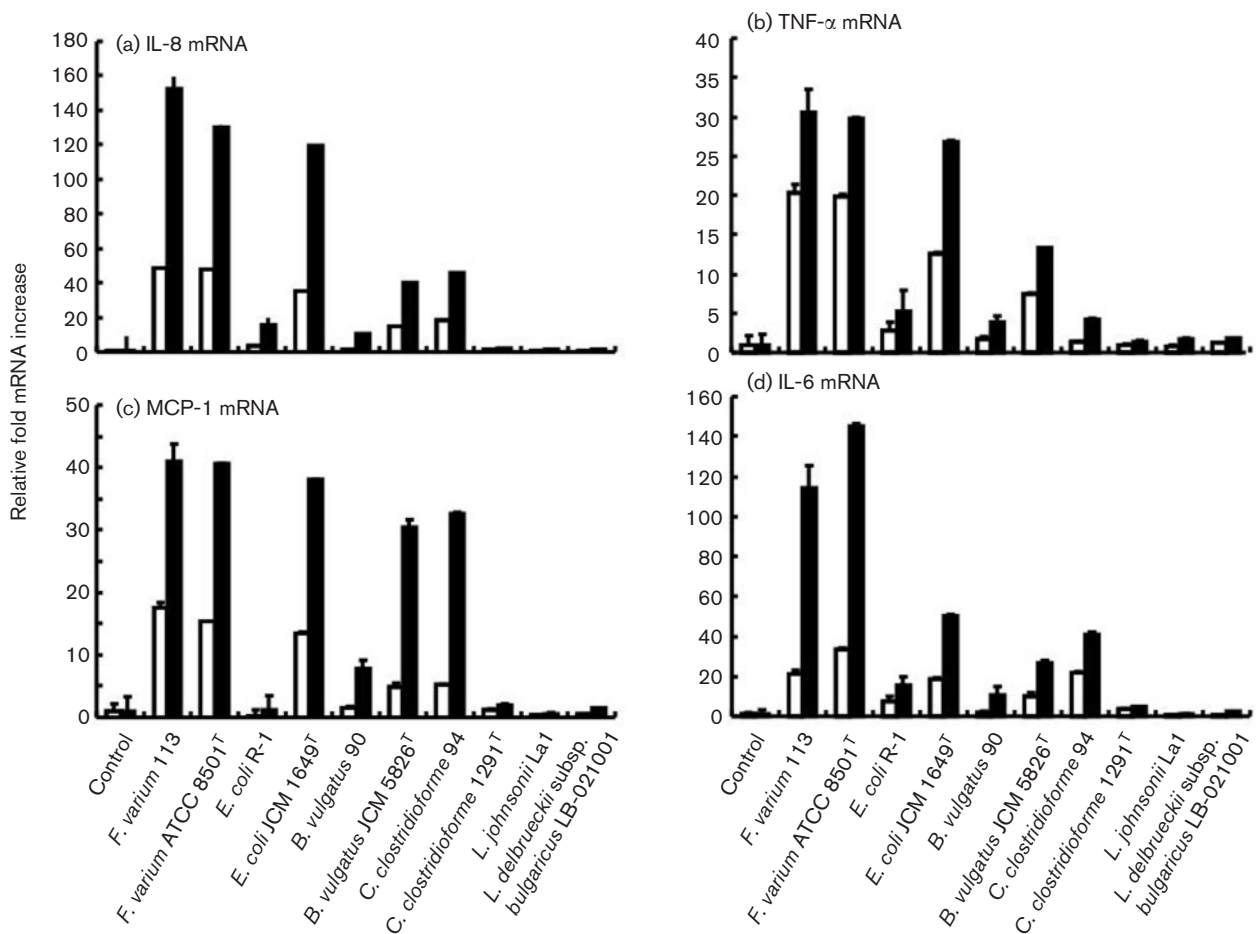
with heterogeneous expression among cell lines being different from that among normal colonic mucosa cells, which usually display IL-18 mRNA and protein in relation to IFN- $\gamma$  and Fas-L-dependent cytotoxicity (Pagès *et al.*, 1999; Paulukat *et al.*, 2001). Noninvasive bacteria, i.e. *C. clostridioforme* JCM 1291<sup>T</sup>, *L. johnsonii* La1 and *L. delbrueckii* subsp. *bulgaricus* LB-021001, slightly stimulated IL-6 mRNA expression.

Thus we found that certain commensal bacteria, which can invade epithelial cells, stimulated IL-8 and TNF- $\alpha$  secretion and correspondingly increased expression of IL-8, TNF- $\alpha$ , MCP-1 and IL-6 mRNA in the invaded epithelial cells. However, there was no enhancement of IL-8 or TNF- $\alpha$  secretion and minimal expression of IL-8, TNF- $\alpha$ , MCP-1 and IL-6 mRNA with noninvasive commensal bacteria and probiotics. Expression of IL-8 and TNF- $\alpha$  mRNAs correlated somewhat with cytokine secretion, indicating definite cytokine production in response to bacterial invasion. However, MCP-1 and IL-6 were below their detection limits, despite considerable mRNA expression being demonstrated by real-time RT-PCR. This discrepancy may be due to the relatively high detection limits for



**Fig. 4.** Induction of cytokine mRNAs in SW-480 cells infected with commensal bacteria and probiotics. Dotted bars, the attachment assay; black bars, the invasion assay. (a) IL-8 mRNA. In the attachment assay, the IL-8 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *E. coli* R-1, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, *B. vulgatus* 90 and probiotics ( $P < 0.05$ ). In the invasion assay, the IL-8 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *B. vulgatus* 90, *C. clostridioforme* JCM 1291<sup>T</sup> and probiotics ( $P < 0.0001$ ). The IL-8 mRNA levels with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> in the invasion assay were significantly higher than those in the attachment assay ( $P < 0.0001$ ). (b) TNF- $\alpha$  mRNA. In the attachment assay, the TNF- $\alpha$  mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *E. coli* R-1, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, *B. vulgatus* 90 and probiotics ( $P < 0.0001$ ). In the invasion assay, the TNF- $\alpha$  mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *C. clostridioforme* JCM 1291<sup>T</sup> and probiotics ( $P < 0.0001$ ). (c) MCP-1 mRNA. In the attachment assay, the MCP-1 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *E. coli* R-1, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, *B. vulgatus* 90 and probiotics ( $P < 0.0001$ ). In the invasion assay, the MCP-1 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *C. clostridioforme* JCM 1291<sup>T</sup>, *E. coli* R-1 and probiotics ( $P < 0.0001$ ). (d) IL-6 mRNA. In the attachment assay, the IL-6 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113 and *E. coli* JCM 1649<sup>T</sup> were significantly higher than those with probiotics ( $P = 0.023 - < 0.0001$ ). In the invasion assay, the IL-6 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *B. vulgatus* 90, *C. clostridioforme* JCM 1291<sup>T</sup> and probiotics ( $P = 0.0081 - < 0.0001$ ).





**Fig. 5.** Induction of cytokine mRNAs in HT-29 cells infected with commensal bacteria and probiotics. White bars, the attachment assay; black bars, the invasion assay. (a) IL-8 mRNA. In the attachment assay, the IL-8 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *E. coli* R-1, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, *B. vulgatus* 90 and probiotics ( $P < 0.01$ ). In the invasion assay, the IL-8 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *B. vulgatus* 90, *C. clostridioforme* JCM 1291<sup>T</sup> and probiotics ( $P < 0.0001$ ). The IL-8 mRNA levels with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> in the invasion assay were significantly higher than those in the attachment assay ( $P < 0.0001$ ). (b) TNF- $\alpha$  mRNA. In the attachment assay, the TNF- $\alpha$  mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *E. coli* R-1, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, *B. vulgatus* 90 and probiotics ( $P < 0.0001$ ). In the invasion assay, the TNF- $\alpha$  mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *C. clostridioforme* JCM 1291<sup>T</sup> and probiotics ( $P < 0.0001$ ). (c) MCP-1 mRNA. In the attachment assay, the MCP-1 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup> and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *E. coli* R-1, *C. clostridioforme* 94 and JCM 1291<sup>T</sup>, *B. vulgatus* 90 and probiotics ( $P < 0.0001$ ). In the invasion assay, the MCP-1 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *C. clostridioforme* JCM 1291<sup>T</sup>, *E. coli* R-1 and probiotics ( $P < 0.0001$ ). (d) IL-6 mRNA. In the attachment assay, the IL-6 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113 and *E. coli* JCM 1649<sup>T</sup> were significantly higher than those with probiotics ( $P = 0.023 - < 0.0001$ ). In the invasion assay, the IL-6 mRNA levels after incubation with *F. varium* ATCC 8501<sup>T</sup> and 113, *E. coli* JCM 1649<sup>T</sup>, *C. clostridioforme* 94 and *B. vulgatus* JCM 5826<sup>T</sup> were significantly higher than those with *B. vulgatus* 90, *C. clostridioforme* JCM 1291<sup>T</sup> and probiotics ( $P < 0.0001$ ).



MCP-1 and IL-6. These results suggest that commensal bacteria can both induce and maintain colonic inflammation.

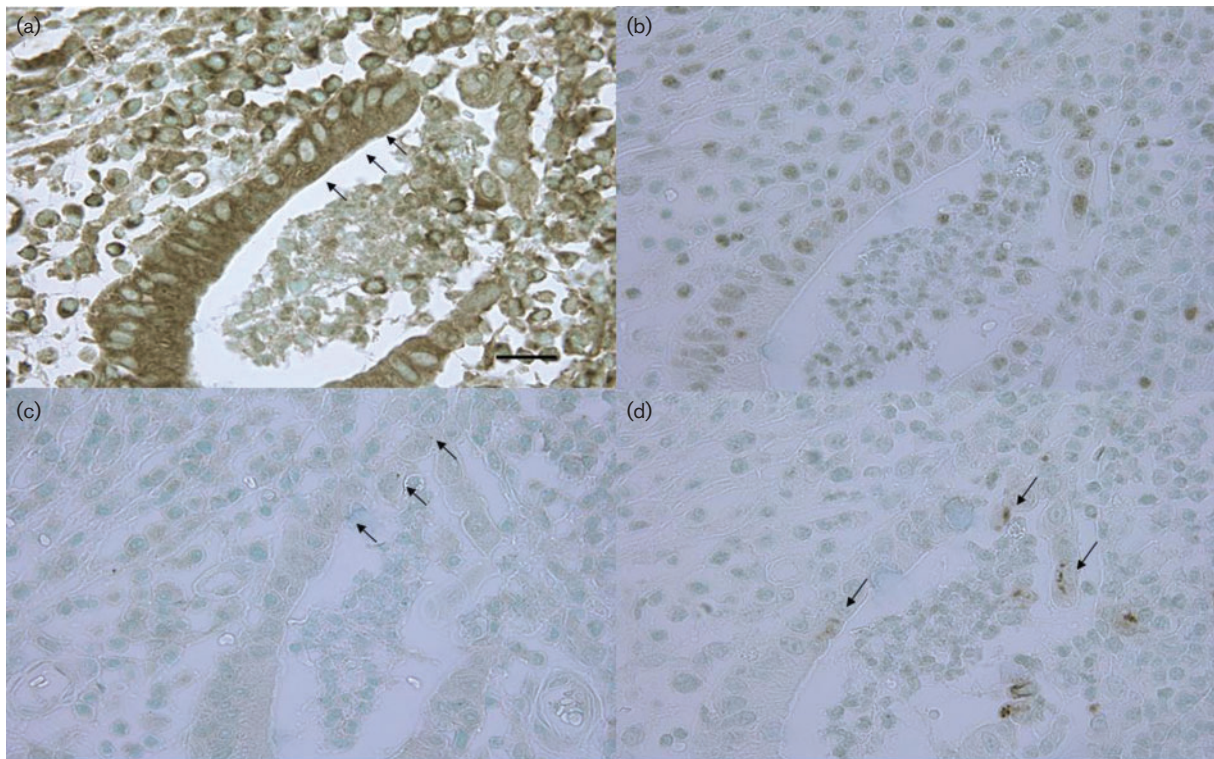
In previous studies, we have shown that bacterial rods and cocci, including *F. varium*, adhered to and invaded the intestinal epithelia and invaded the mucosa propria in patients with UC but not in those with Crohn's disease (Ohkusa *et al.*, 1993, 2002). In this study, we used the type strain and a clinical isolate of *F. varium*; the two strains have almost the same virulence factors, such as enhancement of inflammatory cytokine secretion and invasion of epithelial cells.

### Immunoreactive cytokine expression in inflamed mucosal epithelia in patients with UC

To confirm the results described above by *in vitro* co-culture experiments, we attempted to visualize the immunoreactive expression of TNF- $\alpha$ , IL-8, NF- $\kappa$ B p65 and phosphor-NF- $\kappa$ B p65 in inflamed crypt epithelia with active UC (cryptitis and crypt abscesses) and in rectal epithelia from patients with active UC, using an immunohistochemical method, and results were compared among rectal mucosae of normal controls, inactive UC specimens and active UC specimens. Cytoplasmic granular expression

of TNF- $\alpha$  was extensive in epithelia of half of the crypts with cryptitis and 68% of those with crypt abscesses, while no expression was detected in crypts of normal control or inactive UC samples. Similarly, inflamed epithelium expressed IL-8 in the cytoplasm in active UC specimens but not in crypts of normal control and inactive UC specimens. NF- $\kappa$ B p65 was ubiquitously expressed in the cytoplasm of rectal epithelial cells. However, crypts with epithelia showing nuclear expression were frequently found in active UC specimens, whereas such crypts were rarely seen in normal control and inactive UC samples. Activated phosphor-NF- $\kappa$ B p65 was also frequently found in nuclei of inflamed epithelia in active UC samples, but not in those of normal control and inactive UC samples (Fig. 6, Table 2).

Although epithelia from crypts of normal control or inactive UC samples were not positive for either TNF- $\alpha$  or IL-8, those of active UC samples were positive for both, supporting our proposed pathway. Furthermore, nuclear expression of NF- $\kappa$ B p65 and phosphor-NF- $\kappa$ B p65 was also demonstrated in most of these inflamed crypts, indicating that NF- $\kappa$ B activation is due to stimulation of inflammatory cytokine production and release. In our previous study, *F. varium* invasion was lower in inactive UC (Ohkusa *et al.*, 2002), and bacterial invasion may



**Fig. 6.** Representative photographs of immunohistochemical expression of TNF- $\alpha$ , IL-8, NF- $\kappa$ B p65 and phosphor-NF- $\kappa$ B p65 in semiserial sections of inflamed epithelium featuring cryptitis or crypt abscesses. (a) TNF- $\alpha$  expression in a crypt abscess. Note positive granules in the epithelial cytoplasm (arrows) (bar, 25  $\mu$ m). (b) IL-8 expression in a crypt abscess. Note the positive reaction in the epithelial cytoplasm. (c) NF- $\kappa$ B p65 expression in epithelial nuclei in a crypt abscess (arrows). (d) Phosphor-NF- $\kappa$ B p65 expression in epithelial nuclei in a crypt abscess (arrows).

**Table 2.** Immunoreactive cytokine expression in mucosal epithelium (positive crypts/crypts examined)

Crypts contained at least two positive epithelia.

Rectal mucosa*	TNF- $\alpha$	IL-8	NF- $\kappa$ B p65 in nuclei	Phosphor-NF- $\kappa$ B p65 in nuclei
Normal control	0/20 (0.0%)	0/20 (0.0%)	1/20 (5.0%)	0/20 (0.0%)
UC, inactive	0/24 (0.0%)	0/24 (0.0%)	5/24 (20.8%)	3/24 (12.5%)
UC, active				
Cryptitis	6/12 (50.0%) <sup>†</sup>	10/12 (83.3%) <sup>‡</sup>	12/12 (100.0%) <sup>‡</sup>	12/12 (83.3%) <sup>‡</sup>
Crypt abscess	17/25 (68.0%) <sup>‡</sup>	21/25 (84.0%) <sup>‡</sup>	25/25 (100.0%) <sup>‡</sup>	19/25 (76.0%) <sup>‡</sup>

\*More than 10 cases examined.

<sup>†</sup> $P < 0.0005$  as compared to normal control or inactive UC samples.<sup>‡</sup> $P < 0.0001$  as compared to normal control or inactive UC samples.

spontaneously decrease without eradication therapy in inactive UC. Therefore, epithelia from crypts in inactive UC samples may not be positive for either TNF- $\alpha$  or IL-8.

Previous studies by other investigators have shown that although human intestinal epithelial cell lines constitutively express IL-8 and TGF- $\beta$ 1, other cytokines such as TNF- $\alpha$  are expressed only after challenge with invasive strains of enteropathogenic bacteria (Eckmann *et al.*, 1993a, b). Moreover, expression of TNF- $\alpha$  and IL-8 genes is not altered by incubation with noninvasive *E. coli* or *Enterococcus faecium* (Jung *et al.*, 1995). Adherence is a common characteristic shared by many pathogens since it is a crucial step for establishing an infection (Mann & Petri, 1995). However, we found that noninvasive commensal bacteria and probiotics, despite having the ability to adhere to cells, could not stimulate IL-8 secretion. Therefore, bacterial invasion appears to be a critical factor for the enhancement of secretion and expression of proinflammatory cytokines in colonic epithelial cells.

We conclude that there are harmful commensal bacteria in the colon that can invade the colonic epithelia, induce epithelial production of proinflammatory cytokines, and activate intracellular inflammatory signalling. Therefore, *F. varium* isolated from the colonic mucosa of patients with UC may be among the causative agents of active colitis. Recently, Andoh *et al.* (2007) performed terminal restriction fragment length polymorphism analysis of the faecal microbiota from UC patients and reported that terminal restriction fragments derived from *Fusobacterium* and other unclassified bacteria were predominantly detected in the active UC patients but not in the inactive UC patients and the healthy individuals. These findings have implications for antibiotic therapy against harmful commensal bacteria, aimed at preventing colonic inflammation (Nomura *et al.*, 2005; Ohkusa *et al.*, 2005).

## ACKNOWLEDGEMENTS

This work was supported in part by Grants in Aid for Scientific Research (15590688, 18659102) from the Japanese Ministry of

Education, Culture, Sports, Science and Technology, NIH grants AI09268, GM54666, DE08293 and DE08240, and by grants from the Uehara Memorial Foundation. We thank Malcolm Moore, PhD, for revision of the scientific English language.

## REFERENCES

- Aihara, M., Tsuchimoto, D., Takizawa, H., Azuma, A., Wakebe, H., Ohmoto, Y., Imagawa, K., Kikuchi, M., Mukaida, N. & Matsuhima, K. (1997). Mechanisms involved in *Helicobacter pylori*-induced interleukin-8 production by a gastric cancer cell line, MKN45. *Infect Immun* **65**, 3218–3224.
- Andoh, A., Sakata, S., Koizumi, Y., Mitsuyama, K., Fujiyama, Y. & Benno, Y. (2007). Terminal restriction fragment length polymorphism analysis of the diversity of fecal microbiota in patients with ulcerative colitis. *Inflamm Bowel Dis* **13**, 955–962.
- Burke, D. A. & Axon, A. T. (1988). Adhesive *Escherichia coli* in inflammatory bowel disease and infective diarrhoea. *BMJ* **297**, 102–107.
- Eckmann, L., Kagnoff, M. F. & Fierer, J. (1993a). Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect Immun* **61**, 4569–4574.
- Eckmann, L., Jung, H. C., Schurer-Maly, C., Panja, A., Morzycka-Wroblewska, E. & Kagnoff, M. F. (1993b). Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. *Gastroenterology* **105**, 1689–1697.
- Guarner, F., Casellas, F., Borruel, N., Antolin, M., Videla, S., Vilaseca, J. & Malagelada, J. R. (2002). Role of microecology in chronic inflammatory bowel diseases. *Eur J Clin Nutr* **56** (Suppl. 4), S34–S38.
- Han, Y. W. & Miller, V. L. (1997). Reevaluation of the virulence phenotype of the *inv yadA* double mutants of *Yersinia pseudotuberculosis*. *Infect Immun* **65**, 327–330.
- Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. & Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* **162**, 3749–3752.
- Jung, H. C., Eckmann, L., Yang, S. K., Panja, A., Fierer, J., Morzycka-Wroblewska, E. & Kagnoff, M. F. (1995). A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest* **95**, 55–65.
- Kuhara, T., Iigo, M., Itoh, T., Ushida, Y., Sekine, K., Terada, N., Okamura, H. & Tsuda, H. (2000). Orally administered lactoferrin exerts an antimetastatic effect and enhances production of IL-18 in the intestinal epithelium. *Nutr Cancer* **38**, 192–199.

- Mann, B. J. & Petri, W. A., Jr (1995). The role of microbial adherence factors in gastrointestinal disease. In *Infections of the Gastrointestinal Tract*, pp. 99–105. Edited by M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg & R. L. Guerrant. New York: Raven Press.
- Matsuda, H., Fujiyama, Y., Andoh, A., Ushijima, T., Kajinami, T. & Bamba, T. (2000). Characterization of antibody responses against rectal mucosa-associated bacterial flora in patients with ulcerative colitis. *J Gastroenterol Hepatol* **15**, 61–68.
- Mikami, T., Yoshida, T., Akino, F., Motoori, T., Yajima, M. & Okayasu, I. (2003). Apoptosis regulation differs between ulcerative colitis-associated and sporadic colonic tumors: association with surviving and Bcl-2. *Am J Clin Pathol* **119**, 723–730.
- Mitchell, T. J., Whittaker, S. J. & John, S. (2003). Dysregulated expression of COOH-terminally truncated Stat5 and loss of IL2-inducible Stat5-dependent gene expression in Sezary syndrome. *Cancer Res* **63**, 9048–9054.
- Mitsushashi, J., Mikami, T., Saigenji, K. & Okayasu, I. (2005). Significant correlation of morphological remodeling in ulcerative colitis with disease duration and between elevated p53 and p21WAF1 expression in rectal mucosa and neoplastic development. *Pathol Int* **55**, 113–121.
- Mylonaki, M., Rayment, N. B., Rampton, D. S., Hudspith, B. N. & Brostoff, J. (2005). Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflamm Bowel Dis* **11**, 481–487.
- Nomura, T., Ohkusa, T., Okayasu, I., Yoshida, T., Sakamoto, M., Hayashi, H., Benno, Y., Hirai, S., Hojo, M. & other authors (2005). Mucosa-associated bacteria in ulcerative colitis before and after antibiotic combination therapy. *Aliment Pharmacol Ther* **21**, 1017–1027.
- Ohkusa, T., Okayasu, I., Tokoi, S. & Ozeki, Y. (1993). Bacterial invasion into the colonic mucosa in ulcerative colitis. *J Gastroenterol Hepatol* **8**, 116–118.
- Ohkusa, T., Sato, N., Ogihara, T., Morita, K., Ogawa, M. & Okayasu, I. (2002). *Fusobacterium varium* localized in the colonic mucosa of patients with ulcerative colitis stimulates species-specific antibody. *J Gastroenterol Hepatol* **17**, 849–853.
- Ohkusa, T., Okayasu, I., Ogihara, T., Morita, K., Ogawa, M. & Sato, N. (2003). Induction of experimental ulcerative colitis by *Fusobacterium varium* isolated from colonic mucosa of patients with ulcerative colitis. *Gut* **52**, 79–83.
- Ohkusa, T., Nomura, T. & Sato, N. (2004). The role of bacterial infection in the pathogenesis of inflammatory bowel disease. *Intern Med* **43**, 534–539.
- Ohkusa, T., Nomura, T., Terai, T., Miwa, H., Kobayashi, O., Hojo, M., Takei, Y., Ogihara, T., Hirai, S. & other authors (2005). Effectiveness of antibiotic combination therapy in patients with active ulcerative colitis: a randomized, controlled pilot trial with long-term follow up. *Scand J Gastroenterol* **40**, 1334–1342.
- Pagès, F., Berger, A., Henglein, B., Piqueras, B., Danel, C., Zinzindohoue, F., Thiounn, N., Cugnenc, P. H. & Fridman, W. H. (1999). Modulation of interleukin-18 expression in human colon carcinoma: consequences for tumor immune surveillance. *Int J Cancer* **84**, 326–330.
- Paulukat, J., Bosmann, M., Nold, M., Garkisch, S., Kämpfer, H., Frank, S., Raedle, J., Zeuzem, S., Pfeilschifter, J. & Mühl, H. (2001). Expression and release of IL-18 binding protein in response to IFN-gamma. *J Immunol* **167**, 7038–7043.
- Sadlack, B., Merz, H., Chorley, H., Schimble, A., Feller, A. C. & Horak, I. (1993). Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* **75**, 253–261.
- Sartor, R. B. (2004). Microbial influences in inflammatory bowel disease: role in pathogenesis and clinical implications. In *Kirsner's Inflammatory Bowel Disease*, 6th edn, pp. 138–140. Edited by R. B. Sartor & W. J. Sandborn. Edinburgh: Saunders.
- Sellon, R. K., Tonkonogy, S., Schultz, M., Dieleman, L. A., Grenther, W., Balish, E., Rennick, D. M. & Sartor, B. B. (1998). Resident enteric flora are necessary for development of spontaneous colitis and immune system activation in IL-10-deficient mice. *Infect Immun* **66**, 5224–5231.
- Seo, M., Okada, M., Yao, T., Ueki, M., Arima, S. & Okumura, M. (1992). An index of disease activity in patients with ulcerative colitis. *Am J Gastroenterol* **87**, 971–977.
- Singer, M. & Sansonetti, P. J. (2004). IL-8 is a key chemokine regulating neutrophil recruitment in a new mouse model of *Shigella*-induced colitis. *J Immunol* **173**, 4197–4206.
- Suzuki, M., Hisamatsu, T. & Podolsky, D. K. (2003). Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex. *Infect Immun* **71**, 3503–3511.
- Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., Loening-Baucke, V., Ortner, M., Weber, J., Hoffmann, U., Schreiber, S. & other authors (2002). Mucosal flora in inflammatory bowel disease. *Gastroenterology* **122**, 44–54.
- Vitiello, M., D'Isanto, M., Galdiero, M., Raieta, K., Tortora, A., Rotondo, P., Peluso, L. & Galdiero, M. (2004). Interleukin-8 production by THP-1 cells stimulated by *Salmonella enterica* serovar Typhimurium porins is mediated by AP-1, NF-kappaB and MAPK pathways. *Cytokine* **27**, 15–24.
- Weinstein, D. L., O'Neill, B. L. & Metcalf, E. S. (1997). *Salmonella typhi* stimulation of human intestinal epithelial cells induces secretion of epithelial cell-derived interleukin-6. *Infect Immun* **65**, 395–404.
- Yoshida, T., Haga, S., Numata, Y., Yamashita, K., Mikami, T., Ogawa, T., Ohkusa, T. & Okayasu, I. (2006). Disruption of p53-p53R2 DNA repair system in ulcerative colitis contributes to colon tumorigenesis. *Int J Cancer* **118**, 1395–1403.