Digestive Diseases and Sciences, Vol. 50, No. 8 (August 2005), pp. 1426–1431 (© 2005)
DOI: 10.1007/s10620-005-2857-5

Diallyl Trisulfide Inhibits Tumor Necrosis Factor-α Expression in Inflammed Mucosa of Ulcerative Colitis

AI-PING BAI, MD,* QIN OUYANG, MD,† and REN-WEI HU, MD†

The present study aimed to investigate the effect of diallyl trisulfide (DATS) on tumor necrosis factor (TNF)-α expression in inflammed mucosa of ulcerative colitis and its possible mechanism. Colonic biopsies from ulcerative colitis were treated with 0, 1, 5, and 10 μM DATS for 24 hr. Lactate dehydrogenase (LDH) activities and concentrations of TNF-α in supernatants were measured. mRNA expressions of TNF-α in biopsies were analyzed by RT-PCR. The expression of NF-κB P65 in tissues was studied by immunohistochemistry. Concentrations of TNF-α in supernatants of biopsies treated with 5 and 10 μM DATS were lower than those of untreated biopsies. There were fewer lamina propria mononuclear cells whose NF-κB P65 expression in nuclei was positive, and less mRNA expression of TNF-α in biopsies treated with 10 μM DATS than in untreated biopsies. There were no differences in LDH activities in supernatants between tissues treated with DATS and untreated tissues. DATS could downregulate TNF-α production and inhibit NF-κB activation in lamina propria mononuclear cells of inflammed mucosa, without any effect on the viability of colonic tussue cells.

KEY WORDS: diallyl trisulfide; ulcerative colitis; tumor necrosis factor-α; nuclear factor-κB.

Garlic is a popular foodstuff throughout the world. Garlic extract has been used to treat some diseases such as fungal and bacterial infection in China for a long time ago (1, 2). The properties of garlic extract have been well reported, including antioxidational, antiatherosclerotic, antimutagenic, and anticarcinogenic properties (3–7). For example, garlic extract can ameliorate oxidative stress and preserve the activity of antioxidant enzymes in the kidney, which means that garlic extract has a renoprotective effect in gentamicin-induced acute renal failure in rats (3). Other studies have reported that garlic extracts were potent agents for protecting LDLs against oxidation and glycation, and could benefit patients with diabetes mellitus or cardiovascular diseases by preventing complications (4). Some epidemiological investigations have demonstrated a reverse correlation between gastric cancer incidence and garlic consumption (5), and a double-blind trial in China showed that garlic extract could prevent the progression of precancerous gastric lesions (6). A previous study suggested that treatment with garlic oil can induce antitumorigenic gene expression in human coloretal cancer cells 97). Garlic extract, identified by gas chromatography, has three main components: diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) (8). The three components have similar structures and often share the same biological functions. However, the components, containing different sulfur atoms as their names show, have different biological effectivities: ranked in order, DATS, DADS, DAS (9). DATS is commonly used in more research.

The etiology of ulcerative colitis (UC) is still unclear. It is believed that altered immunological function, resulting from interplay between genetic susceptibility and certain environmental factors, contributes significantly to

Manuscript received October 3, 2004; accepted December 13, 2004.

From the *Department of Gastroenterology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou City 510080, and †Department of Gastroenterology, Westchina Hospital, Sichuan University, Chengdu City 610041, China.

Address for reprint requests: Dr. Ai-Ping Bai, Department of Gastroenterology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou City 510080, China; baiap@163.com.

mucosal inflammation of the gastrointestinal tract (10). During the process of UC, tumor necrosis factor-α (TNF-α) plays a pivotal role in the pathogenesis of altered mucosal immune function (11). Nuclear factor-κB (NF-κB) is the downstream molecule in the TNF-αsignal pathway (12). Activation of NF-κB which is triggered by TNF-α stimulation can regulate expression of proinflammatory cytokines including TNF-α, which forms positive feedback and aggravates the inflammatory response in intestinal mucosa (12). Furthermore, failure of clinical treatment of UC has been related to early reactivation of TNF-α secretory capacity by immunocompetent cells (13). Inhibiting TNF-α secretion in inflammed mucosa of UC is the goal of treating the disease and preventing relapse.

Garlic has been used for treating chronic diarrhea, recorded in ancient Chinese medicine. A similar use has been practiced recently. Treatment with garlic extract and other antiviral agents successfully cured cytomegalovirus enteritis in recipients of allogeneic peripheral blood stem cell transplantation (14). However, there have been no reports on the potential effect of garlic extract on UC. The present study investigated the effect of DATS on TNF-α expression in inflammed mucosa of UC and its possible mechanism.

MATERIALS AND METHODS

Patients. Patients were seven women and five men, with age range 21 to 65 years and median age 35 years. None of the patients received corticosteroids, immunosuppressives, or cytotoxic drugs. The diagnosis of UC had been established by clinical, endoscopic, histologic, and/or radiological criteria. The presence of infection or parasites was excluded by stool culture and microscopic stool examination. Disease activity in UC patients was determined using a grading scale including clinical and paraclinical variables as described in detail (15). Among UC patients, two had mild disease activity, six moderate disease activity, and four severe disease activity. The study was approved by the local Ethics Committee of the University of Sichuan Province.

Tissue Culture. Four to six mucosal biopsies within a 5-cm^2 area of the colonic mucosa without macroscopic lesions were taken from each UC patient by endoscopy. The biopsies were weighed and washed in sterile PBS, pH 7.4, then incubated in RPMI 1640 supplemented with 10% fetal calf serum, 250 U/ml penicillin, 250 μg/ml streptomycin, 10 μg/ml gentamycin, and 0.625 μg/ml fungizone for 1 hr at 37°C. After being washed in sterile PBS, pH 7.4, three times, the specimens were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 20 mM sodium bicarbonate. For 24 hr of culture, the tissues were treated with 0, 1, 5, or 10 μM DATS (Shanghai Hefeng Pharmacy Co., China; 97.98% purity). All tissues were placed in a humidified 5% CO_2 chamber at 37°C. After 24 hr, biopsies were collected and supernatants were stored at −70°C until analysis.

Enzyme-Linked Immunosorbent Assays (ELISAs) for TNF-α. A TNF-α ELISA kit was supplied by Jingmei Biotech Co. Ltd. (China). ELISAs were performed per the manufacturer's instructions. In short, polyclonal goat anti-human TNF-α antibodies were used as capturing antibodies, and biotinylated polyclonal rabbit anti-human TNF-α antibodies as detecting antibody. Streptavidin-HRP and TMBS were added as color indicator. Plates were read at 450 nm right after the color reaction was stopped with acid. All steps were performed at room temperature.

Measurement of LDH Concentration. A lactate dehydrogenase (LDH) concentration measurement kit was purchased from Nanjing Jianchen Biotechnology Inc. (China). LDH concentrations in supernatants were analyzed per the manufacturer's instructions using the spectrophotometric method (16). Tissue viability was assessed based on release of LDH per milligram tissue weight.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RNA from biopies was extracted using Trizol (Life Technologies, Scotland) as described (17). Purified total RNA was quantitated by absorbance at 260 nm. cDNA copy was made from 1 μg of total RNA using a 5 mM concentration of the appropriate antisense oligo(dT) primer for each cDNA synthesis. Two different DNA fragments were amplified from the cDNA generated. A 407-bp DNA fragment of TNF-α was amplified using upstream primer TTAAGCTTTGGCTGAACCGCCGGGCAATGCC and downstream primer CAGGATCCTCACAGGGGAATGATCCCAAAGTA, and a 194-bp fragment of GAPDH as internal quality control using primers CCATGGAGAAGGCTGGGG and CAAAGTTGTCATGGATGACC. PCR reaction was started with a denaturation step at 94°C for 5 min, proceeded with 30 cycles at 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min, and ended with a 10-min extension step. PCR products were separated by 2% agarose gel electrophoresis, visualized following ethidium bromide staining, and semiquantified by comparing the products of TNF-α with that of GAPDH using an imaging densitometer (GS-670; Bio-Rad).

Detection of Nuclear NF-κB P65 by Immunohistochemistry. The biopsies were fixed in 10% buffered formalin and embedded in paraffin; sections of 4 μm were cut. After blocking inner peroxidase, they were sequentially incubated with rabbit anti-human NF-κB P65 (C20; Santa Cruz Biotechnology Inc., USA) at a dilution of 1:500, followed by three washes in PBS, pH 7.4. The sections were then incubated in goat anti-rabbit IgG conjugated to peroxidase-labeled polymer, colored by diaminobenzidine reaction, and counterstained with hematoxylin. Sections were evaluated by light microscopy, and 100 lamina propria mononuclear cells (LPMC) per high-power field (magnification, ×704) were determined for statistical analysis. Cells staining positive for anti-NF-κB (P65) antibody in the cytoplasm and nucleus were counted as NF-κB-positive cells; those with negative staining for anti-NF-κB (P65) antibody in the nucleus were regarded as NF-κB negative cells in biopies. Negative controls were established by omitting the primary antibodies.

RESULTS

LDH Activity. LDH activity in the supernatant of each group after 24-hr culture is shown in Figure 1. There was no difference in LDH activity in supernatants between tissues without DATS treatment and those treated with 1, 5, or 10 μM DATS for 24 hr ($P > 0.05$).

TNF-α Concentration. The TNF-α concentration in the supernatant of each group after 24-hr culture is shown

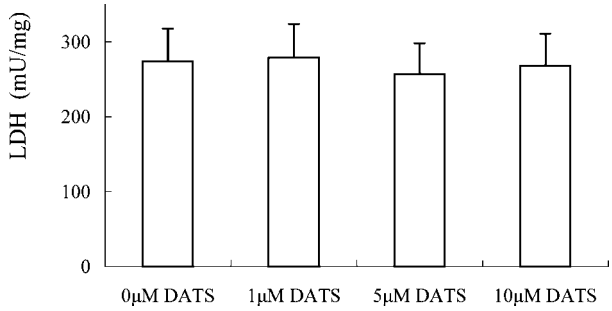


Fig 1. LDH activity in the supernatant of each group. There was no difference in LDH activity in supernatants between tissues without DATS treatment and those treated with 1, 5, or 10 μM DATS for 24 hr ($P > 0.05$).

in Figure 2. The concentration of TNF-α in supernatant of tissues treated with 1 μM DATS was a little lower than that in control tissues, but there was no significant difference between the two group's tissues ($P > 0.05$). However, tissues treated with 5 or 10 μM DATS for 24 hr produced less TNF-α than untreated ($P < 0.05$) vs tissues without DATS treatment.

RT-PCR Analysis. RNA from biopies was extracted and mRNA products were analyzed by RT-PCR. Amplified products were separated on a 2% agarose gel and visualized by ethidium bromide staining. GAPDH was used in the reaction as an internal standard. mRNA expressions of TNF-α and GAPDH in untreated biopsies and those treated with 10 μM DATS are shown in Figure 3. TNF-α product of tissue treated with 10 μM DATS was less than that of tissue without DATS treatment.

Immunuohistochemistry. An immunohistochemical analysis was performed to identify cells with positive staining for anti-NF-κB (P65) antibody in the cytoplasm and the nucleus in biopies without DATS treatment or treated with 10 μM DATS for 24 hr. Cells with positive staining for anti-NF-κB (P65) antibody in the cytoplasm

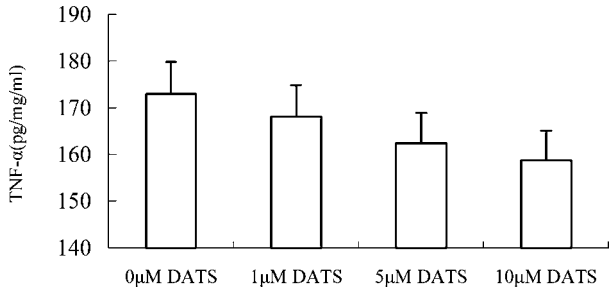


Fig 2. TNF-α concentration in the supernatant of each group after 24-hr culture. There was no difference in TNF-α concentration in supernatant between tissues treated with 1 μM DATS and tissues without DATS treatment, but tissues treated with 5 or 10μM DATS for 24 hr produced less TNF-α than tissues without DATS treatment ($P < 0.05$).

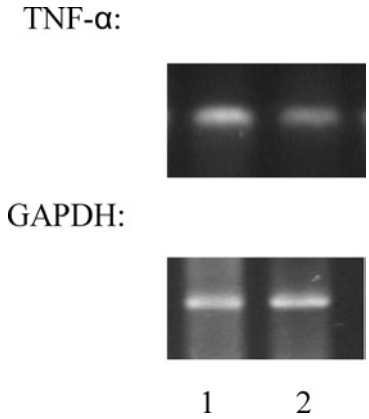


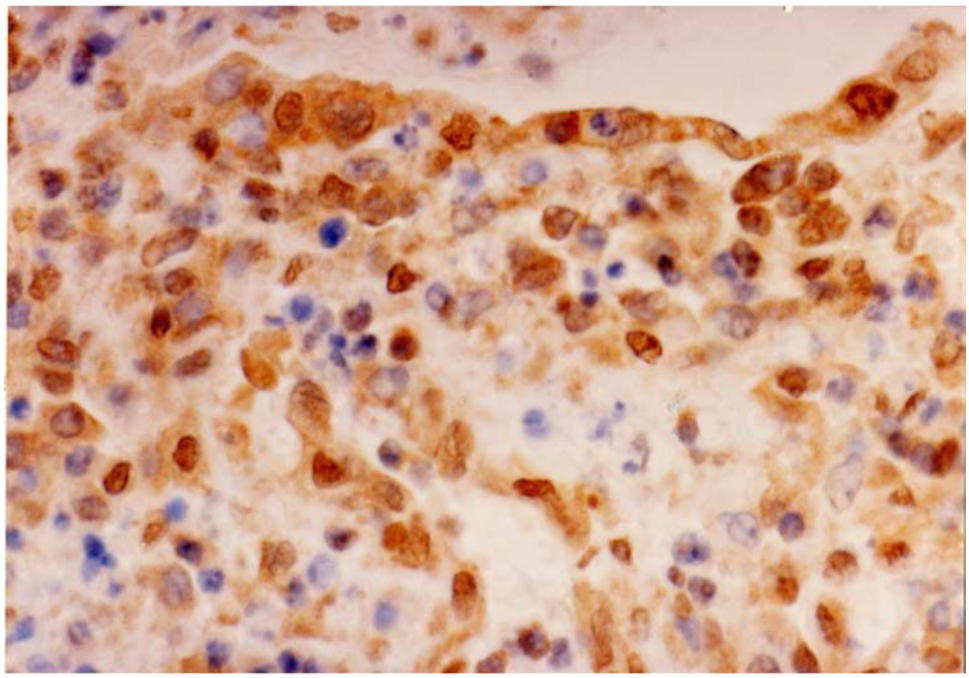
Fig 3. mRNA expression of TNF-α and GAPDH in biopsies without DATS treatment (lane 1) and treated with 10 μM DATS (lane 2). RNA were extracted from biopsies and mRNA products were analyzed by RT-PCR. Amplified products were separated on a 2% agarose gel and visualized by ethidium bromide staining. GAPDH was used in the reaction as an internal standard.

and the nucleus were counted as NF-κB-positive cells; those with negative staining of the anti-NF-κB (P65) antibody in the nucleus were regarded as NF-κB-negative cells in biopies. There were $58.6 \pm 7.3\%$ NF-κB-positive cells in lamina propria of biopsies without DATS treatment (Figure 4A). Treatment with 10 μM DATS resulted in a significant reduction ($P < 0.01$) vs tissues without DATS treatment in NF-κB-positive cells in lamina propria of biopsies: nuclear staining of anti-P65-antibody was observed in only $42.6 \pm 8.5\%$ of cells in the lamina propria (Figure 4B). However, there was no difference in epithelia with nuclear NF-κB detected between biopsies without DATS treatment and those treated with DATS.

DISCUSSION

The principal objective of this study was to determine whether garlic extract could modulate production of TNF-α in colonic biopsies from patients with active UC. TNF-α plays a pivotal role in the pathogenesis of altered mucosal immune function during the process of UC (11, 18). Failure of clinical treatment of UC has been related to early reactivation of TNF-α secretory capacity by immunocompetent cells (13). Inhibiting TNF-α expression in inflammed mucosa of UC is the way to treat the disease and prevent relapse. However, conventional drugs including glucocorticoids, aminosalicyclic acids, and immunosuppressives, are not as effective in curing the disease, as only some patients achieve remission. Some research has explored new drugs to treat the disease, including butyrate (19) and anti-TNF-α antibody (infliximab) (18). In this study, we demonstrated for the first time that 5 or 10 μM DATS can inhibit TNF-α production in colonic biopsies

A



B

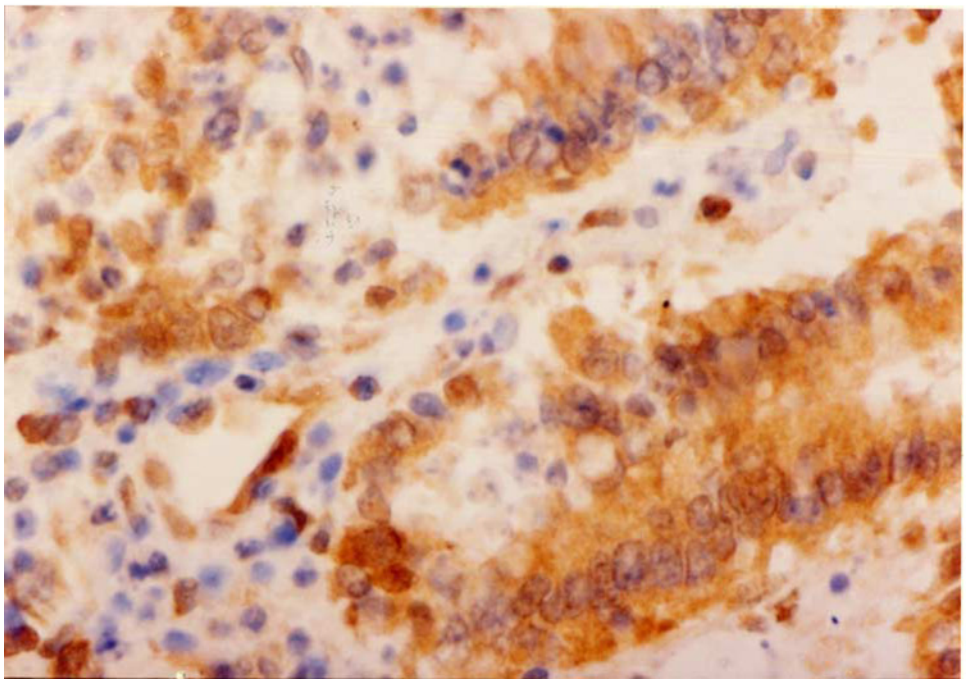


Fig 4. Immunohistochemical staining of sections from colonic biopsies without DATS treatment (A) and treated with 10 μM DATS for 24 hr (B). An anti-NF-κB (P65) antibody was used to detect P65 in the cytoplasm and the nucleus of cells in biopsies. Cells with positive staining for anti-NF-κB (P65) antibody in the cytoplasm and the nucleus were counted as NF-κB-positive cells; those with negative staining for anti-NF-κB (P65) antibody in the nucleus were regarded as NF-κB-negative cells in biopsies. (Original magnification, $\times$704.)

of UC, 10 μM DATS more effectively. DATS may be a potential drug for treatment of UC.

NF-κB activation plays an important role in the regulation of proinflammatory cytokines in IBD (12), including TNF-α, IL-8, and IL-6. In unstimulated immunocompetent cells, NF-κB is confined to the cytoplasm as a homo- or heterodimer bound to a member of the IκB family of inhibitory protein. The majority of NF-κB dimer is composed of the P65 and P50 subunits. When cells receive a stimulus such as TNF-α or IL-1, IκB is phosphorylated and released from the complex, followed by ubiquination and subsequent proteasomal degradation (20). NF-κB then becomes activated, translocates to the nucleus, and binds to the related gene promoter including TNF-α, which forms a positive autoregulatory loop (21). Inhibiting NF-κB activation is the very way to ameliorate the related disease (18). Many effective drugs including glucocorticoids, aminosalicyclic acids, and butyrate can downregulate the inflammatory response in UC by inhibiting the nuclear translocation and the activation of NF-κB. After incubation in 10 μM DATS for 24 hr in the study, biopies show fewer cells with nuclear NF-κB P65 positivity in the lamina propria than without DATS treatment. This means that DATS can successfully inhibit NF-κB activation in lamina propria nuclear cells and downregulate the inflammatory response in mucosa of UC.

Garlic extract has many biological functions which can benefit humans, including antioxidational, antiatherosclerotic, antimutagenic, and anticarcinogenic properties. However, some experimental studies have found that garlic extract showed cytotoxicity. Sundaram *et al.* (22) showed that 100 mM DADS was cytostatic to human tumor cell lines such as HCT-15 (colon), A549 (lung), and SK MEL-2 (skin), and inhibited growth of these cells. Other research demonstrated that apoptosis of tumor cells could be induced by garlic extract (23). When the toxicity of garlic extracts to many nomal cells was discovered, the cytotoxicity and genotoxicity of garlic extract toward normal cells became the focus of investigations. Wu (24) recently demonstrated that a high concentration of DATS (0.05 or 0.25 mM) could affect the viability of rat primary hepatocytes when exposed to different concentrations of DATS for 24 hr, whereas a low concentration of DATS (0.025 mM) did not show any cytotoxicity. In the present study, LDH activity was evaluated as a potential marker of intestinal damage to investigate the toxic effect of DATS on inflammed mucosal tissues by LDH leakage assay (25). There was no difference in LDH leakage between tissues without DATS treatment and those treated with 1, 5, or 10 μM DATS for 24 hr, which means that treatment with a low concentration of DATS did not affect the viability of colonic tissue cells.

In conclusion, DATS can downregulate TNF-α production and inhibit NF-κB activation of lamina propria mononuclear cells in inflammed mucosa of UC, without side effects on the viability of colonic tissue cells. DATS may be a potential drug for treatment of UC.

REFERENCES

1. Xu XQ, Shi SY, Han TZ: A retrospective study of 115 cases of fungal pneumonia. Zhonghua Nei Ke Za Zhi 28:7–10, 1989
2. Tsao SM, Hsu CC, Yin MC: Garlic extract and two diallyl sulphides inhibit methicillin-resistant Staphylococcus aureus infection in BALB/cA mice. J Antimicrob Chemother 52:974–980, 2003
3. Pedraza-Chaverri J, Gonzalez-Orozco AE, Maldonado PD, Barrera D, Medina-Campos ON, Hernandez-Pando R: Diallyl disulfide ameliorates gentamicin–induced oxidative stress and nephropathy in rats. Eur J Pharmacol 473:71–78, 2003
4. Ou CC, Tsao SM, Lin MC, Yin MC: Protective action on human LDL against oxidation and glycation by four organosulfur compounds derived from garlic. Lipids 38:219–224, 2003
5. Fleischauer AT, Poole C, Arab L: Garlic consumption and cancer prevention: meta-analyses of colorectal and stomach cancers. Am J Clin Nutr 72:1047–1052, 2000
6. Gail MH, You WC, Chang YS, Zhang L, Blot WJ, Brown LM, Groves FD, Heinrich JP, Hu J, Jin ML, Li JY, Liu WD, Ma JL, Mark SD, Rabkin CS, Fraumeni JF Jr, Xu GW: Factorial trial of three interventions to reduce the progression of precancerous gastric lesions in Shandong, China: design issues and initial data. Control Clin Trials 19:352–369, 1998
7. Bottone FG Jr, Baek SJ, Nixon JB, Eling TE: Diallyl disulfide (DADS) induces the antitumorigenic NSAID-activated gene (NAG-1) by a p53-dependent mechanism in human colorectal HCT 116 cells. J Nutr 132:773–778, 2002
8. Sundaram SG, Milner JA: Impact of organosulfur compounds in garlic on canine mammary tumor cells in culture. Cancer Lett 74:85–90, 1993
9. Wu CC, Sheen LY, Chen HW, Tsai SJ, Lii CK: Effects of organosulfur compounds from garlic oil on the antioxidation system in rat liver and red blood cells. Food Chem Toxicol 39:563–569, 2001
10. Oliva-Hemker M, Fiocchi C: Etiopathogenesis of inflammatory bowel disease: the importance of the pediatric perspective. Inflamm Bowel Dis 8:112–128, 2002
11. Breese EJ, Michie CA, Nicholls SW, Murch SH, Williams CB, Domizio P, Walker-Smith JA, MacDonald TT: Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. Gastroenterology 106:1455–1466, 1994
12. Neurath MF, Fuss I, Schurmann G, Pettersson S, Arnold K, Muller–Lobeck H, Strober W, Herfarth C, Buschenfelde KH: Cytokine gene transcription by NF-kappa B family members in patients with inflammatory bowel disease. Ann NY Acad Sci 859:149–159, 1998
13. Nikolaus S, Raedler A, Kuhbacker T, Sfikas N, Folsch UR, Schreiber S: Mechanisms in failure of infliximab for Crohn's disease. Lancet 356:1475–1479, 2000
14. Xu L, Guo N, Ren H: Cytomegalovirus enteritis in recipients of allogeneic peripheral blood stem cell transplantation. Zhonghua Nei Ke Za Zhi 40:546–549, 2001
15. Tvede M, Bondesen S, Nielsen OH, Rasmussen SN: Serum antibodies to Bacteroides species in chronic inflammatory bowel disease. Scand J Gastroenterol 18:783–789, 1983

16. Krieg AF, Gorton S, Henry JB: Effect of temperature on activity and lactate optima of LDH isoenzymes. Clin Chim Acta 17:363–366, 1967
17. Chomczynski P, Mackey K: Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques 19:942–945, 1995
18. Sandborn WJ, Targan SR: Biologic therapy of inflammatory bowel disease. Gastroenterology 122:1592–1608, 2002
19. Hove H, Mortensen PB: Influence of intestinal inflammation (IBD) and small and large bowel length on fecal short-chain fatty acids and lactate. Dig Dis Sci 40:1372–1380, 1995
20. Thanos D, Maniatis T: NF-kappa B: a lesson in family values. Cell 80:529–532, 1995
21. Lee JI, Burckart GJ: Nuclear factor kappa B: important transcription factor and therapeutic target. J Clin Pharmacol 38:981–993, 1998
22. Sundaram SG, Milner JA: Diallyl disulfide inhibits the proliferation of human tumor cells in culture. Biochim Biophys Acta 1315:15–20, 1996
23. Filomeni G, Aquilano K, Rotilio G, Ciriolo MR: Reactive oxygen species-dependent c-Jun NH2-terminal kinase/c-Jun signaling cascade mediates neuroblastoma cell death induced by diallyl disulfide. Cancer Res 63:5940–5949, 2003
24. Wu CC, Lii CK, Tsai SJ, Sheen LY: Diallyl trisulfide modulates cell viability and the antioxidation and detoxification systems of rat primary hepatocytes. J Nutr 134:724–728, 2004
25. Oberle RL, Moore TJ, Krummel DA: Evaluation of mucosal damage of surfactants in rat jejunum and colon. J Pharmacol Toxicol Methods 33:75–81, 1995