The Effect of Local Attachment of Cationized Antioxidant Enzymes on Experimental Colitis in the Rat

Sigal Blau,[1] Ron Kohen,[1,3,4] Paul Bass,[2] and Abraham Rubinstein[1,3,4]

Received May 21, 2000; accepted June 13, 2000

Purpose. To investigate the possibility of local treatment of colitis with the adhesive antioxidant enzymes catalase and superoxide dismutase (SOD).

Methods. The net electric charge of the enzymes' surfaces was modified from negative to positive, to cause their adherence to the colon epithelium. The effects of this local administration were assessed in inflamed rat colon. Inflammation severity (colitis) was assessed by measuring colonic tissue myeloperoxidase (MPO) activity, amounts of tumour necrosis factor alpha (TNFα) and concentrations of reduced glutathione (GSH). The measurements were carried out in two types of protocols: preventive (pre-colitis induction) and treatment (post-colitis induction). In addition, the efficacy of treatment with the cationized enzymes was compared to 5-aminosalicylic acid (5-ASA) and betamethasone with similar administration routes.

Results. The two cationized antioxidant enzymes were found to be efficient in both prevention and treatment of experimental colitis. The two cationized enzymes caused a significant reduction in MPO activity. A reduction in TNFα concentration was noted only after the treatment protocol. No correlation was found between inflammation severity and tissue levels of GSH. In most cases the cationized enzymes were more effective than 5-ASA and betamethasone.

Conclusion. Cationized catalase and cationized SOD have the potential to be efficient therapeutic tools in the local treatment of colitis.

KEY WORDS: experimental colitis; adhesive antioxidant enzymes; cationization; superoxide dismutase (SOD); catalase; local attachment; 5-aminosalicylic acid (5-ASA); betamethasone.

INTRODUCTION

The pathophysiology of inflammatory bowel disease (IBD) is characterized by the involvement of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, and hypochlorous acid (1). The tissue damage caused by ROS is a result of either direct damage to proteins and other cellular macromolecules, or indirect damage caused by upregulation of cytokine production. ROS produced by luminal sources are also suggested to be involved in the sudden exacerbation of IBD (2).

The involvement of ROS in the pathophysiology and pathogenesis of IBD suggests that an enrichment of the colonic tissue with antioxidants could reduce the oxidative damage they cause. The two antioxidant enzymes, superoxide dismutase (SOD) and catalase, are obvious candidates for that purpose. Previous studies have shown that systemic treatment with SOD reduced the severity of inflammation in experimental colitis, as well as in Crohn's disease patients (3,4). However, the major drawback of systemic administration of antioxidant enzymes like SOD is their ultra-short elimination half-life. Also, the damage induced by ROS is characterized by site-specific events. Therefore, if antioxidant enzymes are to be used for the treatment of IBD, they should be located in close proximity to the site of inflammation. For these reasons, local administration of the antioxidant enzymes is highly favourable. In this study, we hypothesized that the attachment of antioxidant enzymes to the site of inflammation would increase the efficacy of IBD therapy with the enzymes. Such localization can be accomplished by attachment of the modified enzymes to the inflamed tissues. Since catalase and SOD are proteins with a negatively charged surface, one possible way for such an attachment to occur is to change their electric charge.

The advantage of cationized catalase over the native enzyme has been demonstrated in several systems. It was shown that cationized catalase was capable of protecting *E. Coli* cells and cultured endothelial cells (5) against oxidative stress. Moreover, cationized antioxidant enzymes were found to be effective *in vivo* in preventing experimental arthritis in the rat (6). In previous studies, we reported that cationization of catalase significantly increases its ability to prevent tissue damage caused by hydroxyl radicals, hydrogen peroxide, and superoxide radicals when applied topically in the small intestine of the rat (7,8). Consequently, we further postulate that cationized catalase and cationized SOD, if adhered to the intestinal tissue, would be a novel therapeutic method for the treatment of experimental colitis.

The goals of the present study were: (a) to cationize catalase and SOD and maintain their activity, (b) to verify that the modified enzymes adhere better than the native ones to the colonic mucosa of the rat, and (c) to examine if intracolonic administration of the cationized antioxidant enzymes—in two different treatments, pre- and post- colitis induction—is superior to the identical administration of native (non-cationized) enzymes in the management of experimental colitis.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma Chemical Co., St. Louis, MO, USA, unless otherwise mentioned in the text. Commercial preparations of 5-aminosalicylic acid (5-ASA) (Rafassal retention enema) and sennoside A and B (X-prep) (both from Rafa, Jerusalem, Israel) and Betamethasone (Betnasol retention enema, Glaxo, England) were used.

Animals, Maintenance and Euthanasia

Male Sprague–Dawley rats (200-250 g) obtained from the Animal Farm of Hadassah Medical Center and The Hebrew University were kept under constant environmental conditions (22°C, 12 h light/dark cycles) and fed with standard laboratory chow and tap water.

[1] The Hebrew University of Jerusalem, Faculty of Medicine, School of Pharmacy, P.O. Box 12065, Jerusalem 91120, Israel.

[2] The University of Wisconsin, School of Pharmacy, 425 North Charter St., Madison Wisconsin 53706.

[3] Also affiliated with the David R. Bloom Center of Pharmacy at the Hebrew University.

[4] To whom correspondence should be addressed. (e-mail: avri@cc.huji.ac.it; ronko@cc.huji.ac.il)

0724-8741/00/0900-1077$18.00/0

All animal studies were conducted in accordance with the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985). The Mutual Committee of Hadassah University Hospital and the Faculty of Medicine for Animal Welfare approved the study protocol. Euthanasia of the anesthetized rats was carried out by chest wall puncturing.

Cationization of Catalase, SOD, and BSA

In separate experiments, the carboxyl groups of catalase (EC 1.11.1.6), SOD (EC 1.15.1.1), BSA, or Fluoresceine isothiocyanate (FITC)-stained BSA (F-BSA) were substituted with 1,6-diaminohexane in the presence of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl as described previously (7,8).

Characterization of the Cationized Antioxidants Enzymes

Net Molecular Charge. This was assessed qualitatively on a cation exchange column (CM Sephadex) (Table I).

Amine Substitution Assay. The number of primary amine groups was evaluated by fluorescamine reaction as previously reported (7). The slopes of the linear plots of fluorescence versus enzyme concentrations were then calculated (Table I). Thus, the higher the value of the calculated slope, the higher the levels of primary amines in each protein (i.e., higher degree of cationization).

Enzyme Activity Measurements. The activity of catalase or cationized catalase was determined according to the procedure described by Aebi (9). The activity of SOD or cationized SOD was determined according to the procedure described by McCord and Fridovich (10) (Table I). The measured activity values found were then used to calculate and administer equivalent doses of native or cationized enzymes to the colon of the rats.

Epithelial Attachment Measurements

Qualitative Evaluation. Fluoresceine isothiocyanate (FITC)-stained BSA (F-BSA) served as a model protein for the two cationized enzymes in this study. The colons of Male Sprague–Dawley rats were removed (see below), rinsed with ice-cold phosphate-buffered-saline (PBS) (pH 7.4), and everted on a glass rod. Two everted sacs were prepared from each colon and incubated separately with cationized F-BSA (CF-BSA) or F-BSA for 10 minutes. After incubation, the tissues were rinsed twice with PBS, cut open longitudinally, embedded in OCT compound and stored at –20°C. Ten-micron specimens were cut for microscopic analysis with cryostat.

Quantitative Evaluation. The total activity of tissue SOD and catalase was used to compare the adherence of the cationized and native enzymes to the colon epithelium of the rat. The colons of Male Sprague–Dawley rats were removed (see below), cut open, rinsed with ice-cold PBS, pH 7.4, and divided into 6–10 segments, 1 cm long each. The segments were incubated for 10 minutes with cationized or native enzymes (catalase or SOD) or PBS and then rinsed with PBS to get rid of excess of non-adhered enzymes. To compare the mucosal adherence capabilities of the various enzymes, the rinsed colonic segments were then shaken separately in 5 ml of the same buffer, at 37°C for 0, 1, and 4 hours. After incubation, the mucosa of each colonic segment was carefully scraped with a scalpel and homogenized in 0.5 ml of 0.02 M phosphate-buffer, pH 7.4, at 4°C. The tissue homogenates were stored at –74°C until analysis of total SOD and catalase activity (see above). The results were normalized to tissue protein.

Table I. The Degree of Cationization of Catalase and SOD as Calculated from the Ratio of the Slopes of the Fluorescamine Reaction. Their Net Charge as Evaluated by Binding to Cation Exchange Column and Their Activity After Cationization

Compound	Florescamine reaction slope (number of primary amine groups/ μg protein) $\times 10^{15}$	Florescamine reaction slopes ratio (cationized/ native)	Net charge	Cationized enzyme activity (% of native enzyme)
Catalase				
Native	0.8		–	
Cationized	1.7	1.3	+	36
SOD				
Native	1.6		–	
Cationized	5.1	3.3	+	38.2
BSA				
Native	0.8		–	
Cationized	1.1	1.4	+	

Induction of Experimental Colitis

Twenty-four hours prior to colitis induction the rats were deprived of food, but allowed free access to water. The water contained the laxative sennoside A and B (10 mg/l) and sucrose (200 g/l). With the rats under light ether inhalation anaesthesia, colitis was induced by intracolonic administration of 30 mg of dinitrobenzensulfonic acid (DNBS) dissolved in 1 ml of ethanol 25 % (v/v) (11). The solution was instilled slowly over 20 seconds via a flexible, perforated Foley catheter which was then immediately removed, leaving the rats in an upside down position for another 40 seconds.

Local Treatment with Cationized Catalase, Cationized SOD, 5-ASA, and Betamethasone

The effect of the cationized enzymes on experimental colitis was compared to the effect of the native enzymes in two different protocols: (a) pre-colitis induction treatment (Treatment A), which was intended to mimic a treatment in the inactive state of colitis, (b) post-colitis induction treatment (Treatment B), which was intended to mimic a treatment in an active state of colitis. The effect of 5-ASA and betamethasone was assessed using the same two treatment protocols. In Treatment A, in separate studies, Sprague–Dawley rats were dosed intracolonically with 1 ml of cationized catalase solution (activity: 4,400U), or 1 ml of cationized SOD solution (activity: 900U), or 67 mg/ml 5-ASA, or 50 μg/ml betamethasone. The administration was carried out 0.5 hours prior to the induction of colitis and the rats were sacrificed 24 hours after the colitis induction. In Treatment B, in separate studies, rats were dosed intracolonically with 1 ml of cationized catalase solution (activity: 9,600U), or 1 ml of cat-

ionized SOD solution (activity: 1,600U), or 67 mg/ml 5-ASA, or 50 μg/ml betamethasone. The administration was carried out 1 hour following colitis induction and repeated every 12 hr over three days. The dosing regimen in Treatment B was based on preliminary studies in which inflammation was observed within 1-hour post induction (data not shown). The rats were sacrificed 4 days after colitis induction.

Tissue Collection

The rats were anesthetized with Equitensine solution (containing 2% w/v pentobarbitol) and their colons were exposed through a longitudinal abdominal incision. The distal 10-cm of the colon was removed, cut open, and rinsed with ice-cold PBS, pH 7.4. Ulcerated regions were located, and full thickness of ulcerated tissues was separated with a scalpel from the surrounding inflamed tissues. In a related study, the macroscopic identification of the ulcerated regions was verified histologically, indicating a severe ulceration (data not shown). In that same study, although no ulcers were present in the inflamed regions, infiltration of inflammatory cells and oedema was observed. The tissue specimens were immediately frozen in liquid nitrogen. At a later stage the specimens were homogenized (Polytron, Kinematica GmbH, Germany) in 10 volumes of 0.02M phosphate-buffer, pH 7.4, and stored again at –74°C for further biochemical analysis and inflammatory markers' evaluation. Euthanasia of the anesthetized rats was performed by chest wall puncturing.

Quantification of Colonic Inflammation

Myeloperoxidase (MPO) Activity Analysis. MPO activity is a reliable index of inflammation caused by infiltration of activated neutrophils into the colonic tissues. Its activity was evaluated according to Grisham *et al.* (12).

Tumour Necrosis Factor Alpha (TNFα) Tissue Concentrations. These were measured using sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Genzyme Co., Cambridge, MA, USA). To prevent TNFα proteolysis, a mixture of protease inhibitors [PMSF (1mM), aprotinin (10μg/ml), and leupeptin (1μg/ml)] were added to homogenate before the assay.

In both cases (MPO activities and TNFα concentrations) the results obtained were expressed as a fraction (in percentage) of the measurements taken in saline treated rats (controls). Thus, in Treatment A, the activity of MPO in the colitis induced colonic tissues averaged 10.4±1.0 U/g protein, and the concentration of TNFα averaged 0.47±0.03 ng/g protein. In Treatment B, the activity of MPO in the colonic tissues averaged 12.0±2.2 U/g protein, and the concentration of TNFα averaged 0.75±0.08 ng/g protein.

Tissue Levels of Reduced Glutathione (GSH)

The GSH concentration was measured to assess the effect of the antioxidant enzymes on the natural occurring antioxidant GSH. For this purpose, pre-column derivatization of GSH was performed by reaction with *o*-phthalaldehyde (OPT) and the resulted fluorophor concentration was measured with HPLC equipped with a fluorescent detector (13).

The results obtained were expressed as a fraction (in percentage) of the measurements taken in saline treated rats (controls). Thus, in Treatment A and Treatment B the concentration of GSH averaged 4.9±0.9 and 1.5±0.2 μmole/g protein, respectively. In naive rats the concentration of GSH averaged 12.3±1.8 μmole/g protein.

Tissue Protein

Tissue protein was measured by the Bradford method and the results were expressed in mg protein (14).

Statistical Analysis

The results were expressed as mean values ± S.E.M. The Kruskal–Wallis test (15) was used to identify differences among the results obtained following the treatments with the antioxidant enzymes. The Mann–Whitney test was used to identify differences between the results obtained following treatment with 5-ASA and betamethasone compared to the saline treated group. In both cases $p < 0.05$ was considered to be significant.

RESULTS

Characterization of the Cationized Antioxidants Enzymes

The surface charge conversion from negative to positive at physiological pH was verified qualitatively by cation exchange and shown in Table I. Cationization was quantified by assessing the degree of substitution of primary amino groups on the surface of the proteins with the fluorescamine method. The extent of enzyme cationization was calculated by dividing the slopes of the curves of the cationized proteins by the slopes of the curves of the native proteins. Table I shows that in all cases the amount of the primary amino groups was higher following cationization. Its also shows that (a) SOD cationization was more efficient than that of the other two proteins and (b) approximately one third of the enzymes' activity was retained after cationization.

Protein Adherence to Epithelium of the Colon

Microscopic visualization of the adherence of the model probe F-BSA to the colonic epithelium is shown in Figure 1. Large amounts of the CF-BSA covered the mucosa surface. No adherence was observed when the non-cationized F-BSA was examined (data not shown).

Quantitative analysis of the extent of attachment of the cationized catalase and the cationized SOD to the colonic mucosa of the rat is shown in Figure 2. Analysis of the total mucosal activity of the enzymes revealed that the native enzymes were unable to adhere to the tissues while the cationized catalase adhered for at least 1 hour and the cationized SOD for over 4 hours.

Pre-Colitis Induction Enzyme Treatment (Treatment A)

Treatment A with cationized catalase and SOD significantly attenuated the inflammatory response caused by DNBS, as assessed by MPO activity measurements, in the inflamed (non-ulcerated) colonic tissue. No significant effect could be detected when the native (non-cationized) enzymes were used. Figure 3A shows that, following cationized catalase, MPO activity in the rat colonic tissue was reduced sig-

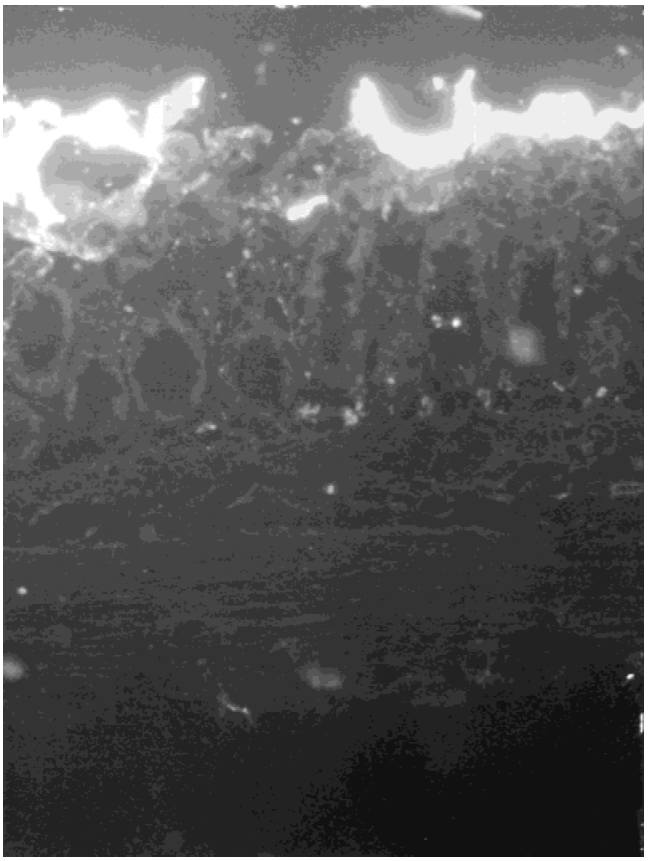

Fig. 1. A microphotograph of CF-BSA attached to the surface of colonic mucosa of the rat.

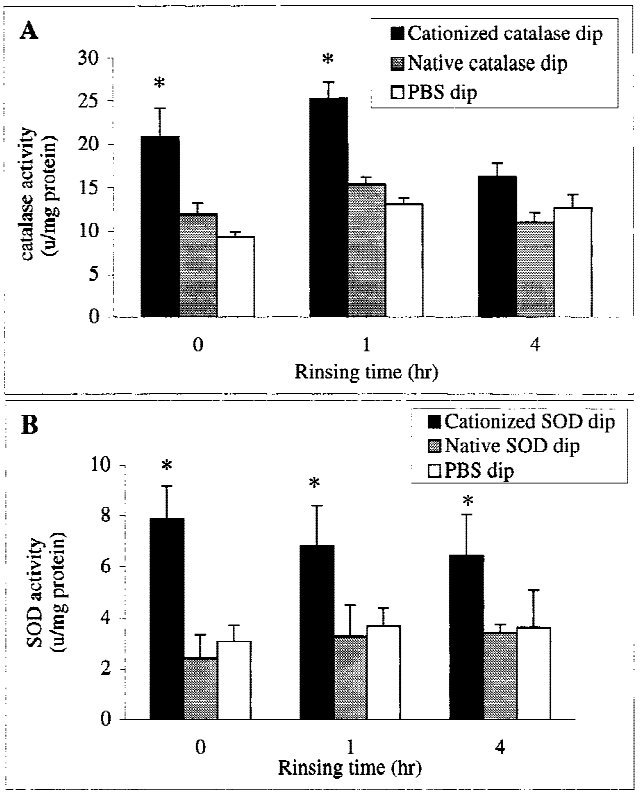

Fig. 2. The attachment of cationized and non-cationized catalase (A) and SOD (B) to the colonic mucosa of the rat as assessed by measuring the overall tissue activity of the enzymes. Shown are the mean values of at least 4 rats ± SEM. Tissues were dipped in saline containing cationized or native enzymes or in PBS (control) for 10 minutes. The tissues were then rinsed (i.e. incubated in saline) for 1 or 4 hours. * $p < 0.05$ compared to native enzymes or PBS.

nificantly more than the reduction observed with native catalase (reduction in activity of 44% and 24%, respectively, compared with the activity measured in tissues taken from saline control treatments). It can also be seen that, following treatment with cationized SOD, reduction in the same order of magnitude (31%) in tissue MPO activity was obtained in the inflamed tissues when compared to MPO activity following native SOD or saline control treatments. Figure 3A also shows the tissue MPO activity after treatment with BSA. The enzyme activities were similar after BSA, cationized BSA, or saline treatments. These findings prove that the protecting effect observed was not a result of the change in the surface electric charge of the enzymes.

Treatment A with the antioxidant enzymes did not cause significant changes in the tissue TNFα concentrations in the inflamed colonic tissue (Figure 3B). It is noteworthy that no difference in MPO activity and TNFα concentrations was observed among the study groups when the ulcerated tissues wre examined (data not shown). It was also observed that the treatment did not reduce either the area of the ulcerated regions or the total tissue we weight. The effect of the antioxidant enzymes on the tissue GSH concentration is shown in Figure 3C. It can be seen that following treatments with SOD (both cationized and native), GSH levels were 5-fold higher compared to the saline control group. It was also found that GSH levels in the colonic tissue were similar to the GSH levels in the naive rats (data not shown). Figure 3C also shows that in Treatment A cationized catalase was unable to prevent GSH reduction.

Post-Colitis Induction Enzyme Treatment (Treatment B)

Treatment B with cationized enzymes caused attenuation in the inflammatory response in the inflamed region, as assessed by both tissue MPO activity and TNFα (Figure 4). Cationized catalase caused a reduction of 39% in tissue MPO activity (Figure 4A) and 33% reduction in tissue TNFα concentration (Figure 4B) compared with the saline treatment controls. A 46% reduction in tissue MPO activity (Figure 4A) and 38% reduction in tissue TNFα concentration (Figure 4B) were observed after treatment with cationized SOD compared to the saline treatment controls. In contrast to Treatment A, cationized catalase, cationized SOD, and the related native enzymes were all able to moderate the reduction of GSH in Treatment B as shown in Figure 4C. Similar to the results found in Treatment A, no difference in MPO activity and TNFα concentration among specimens collected from ulcerated regions could be observed (data not shown). Moreover, as in Treatment A, the cationized enzymes administered post-colitis induction did not reduce either the area of the ulcerated regions or the total tissue wet weight (data not shown).

5-ASA and Betamethasone Treatments

Treatment A conducted with betamethasone and with 5-ASA produced only a slight lowering of MPO activity (after betamethasone) and TNFα tissue levels (after both betameth-

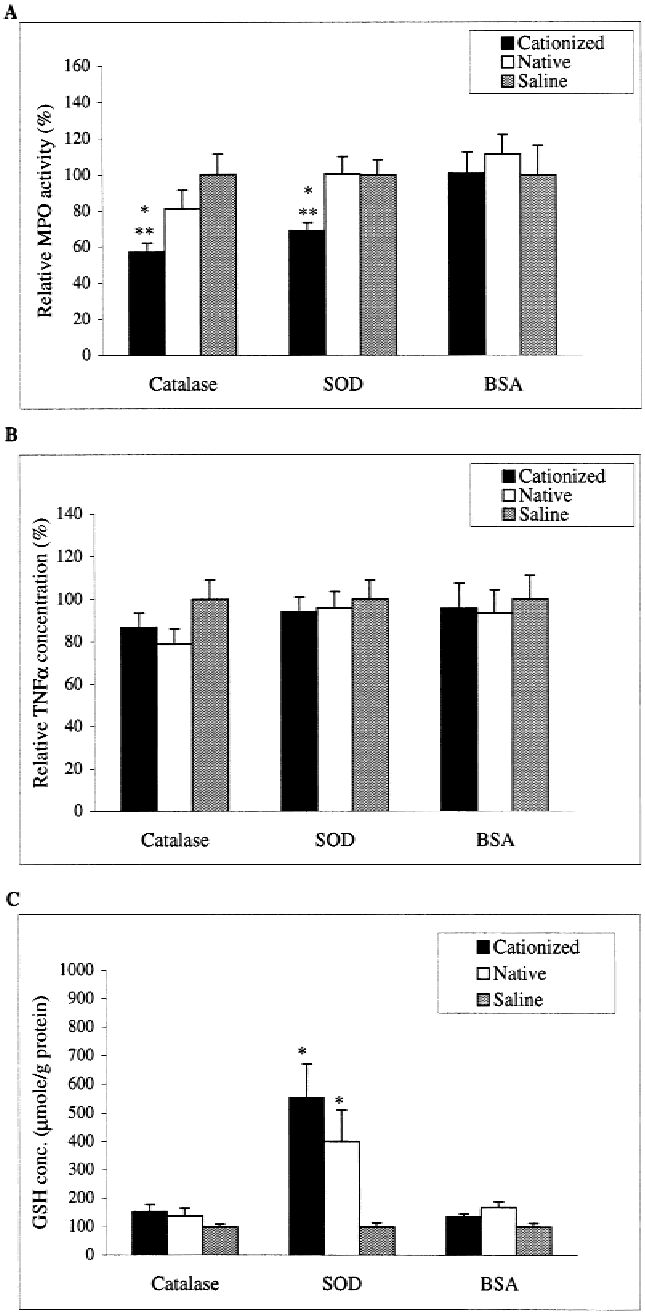

Fig. 3. The effect of cationized and native catalase, SOD or BSA on the severity of colon inflammation and tissue antioxidant status as assessed in non-ulcerated tissue specimens by measuring tissue MPO activity (A), tissue TNFα concentrations (B) and tissue GSH concentrations (C) following Treatment A. Results are expressed as a fraction (in percentage) of saline controls. Shown are the mean values of at least 10 rats ± SEM. * $p < 0.05$ compared to saline treatment. * * $p < 0.05$ compared to native enzymes treatment.

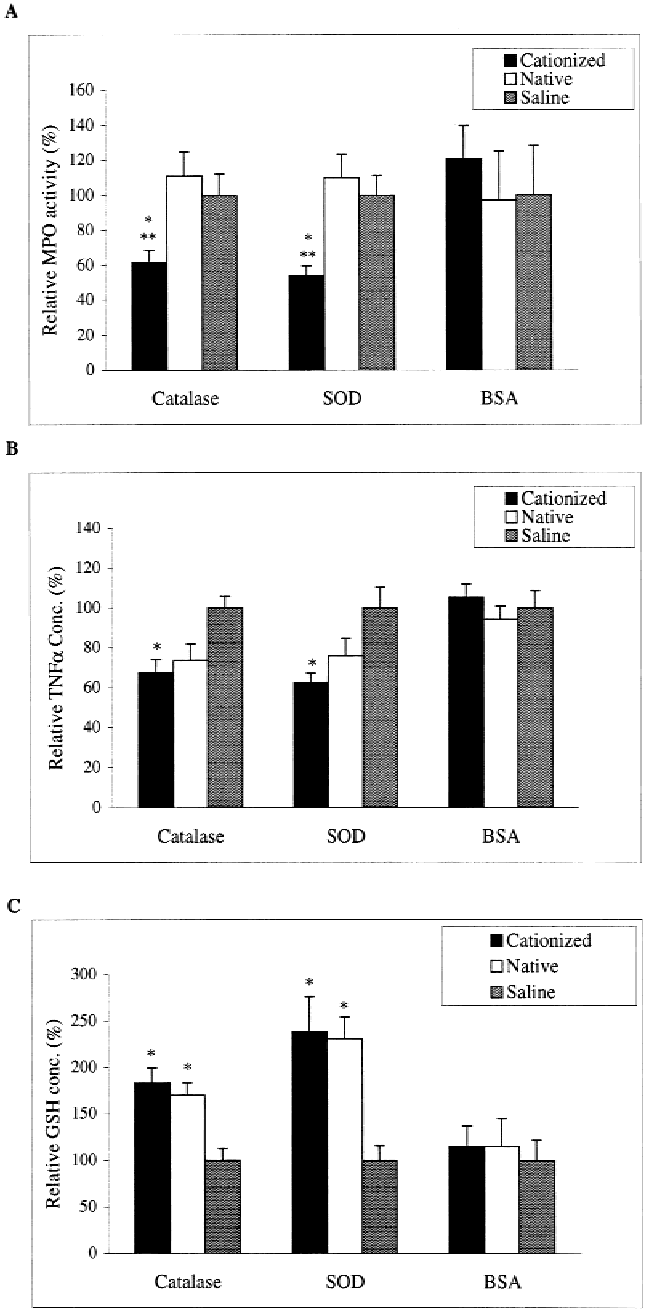

Fig. 4. The effect of cationized and native catalase, SOD or BSA on the severity of colon inflammation and tissue antioxidant status as assessed in non-ulcerated tissue specimens by measuring tissue MPO activity (A), tissue TNFα concentrations (B) and tissue GSH concentrations (C) following Treatment B. Results are expressed as a fraction (in percentage) of saline controls. Shown are the mean values of at least 10 rats ± SEM. * $p < 0.05$ compared to saline treatment. * * $p < 0.05$ compared to native enzymes treatment.

asone and 5-ASA) (Figures 5A, 5B). Nevertheless, 5-ASA was able to prevent tissue GSH depletion while betamethasone was unable to do so (Figure 5C). As in the case of cationized SOD (Figure 3C), Treatment A with 5-ASA caused GSH levels to exceed the basal levels obtained in the naive control experiments. After Treatment B, betamethasone significantly lowered MPO activity and TNFα tissue levels. 5-ASA also resulted in a significant reduction in TNFα concentration, but no significant change in MPO (Figures 5A, 5B) Both drugs were able to inhibit significantly the depletion of tissue GSH (Figure 5C).

DISCUSSION

An increasing number of reports on the involvement of ROS in IBD (16,17) and changes in endogenous antioxidants

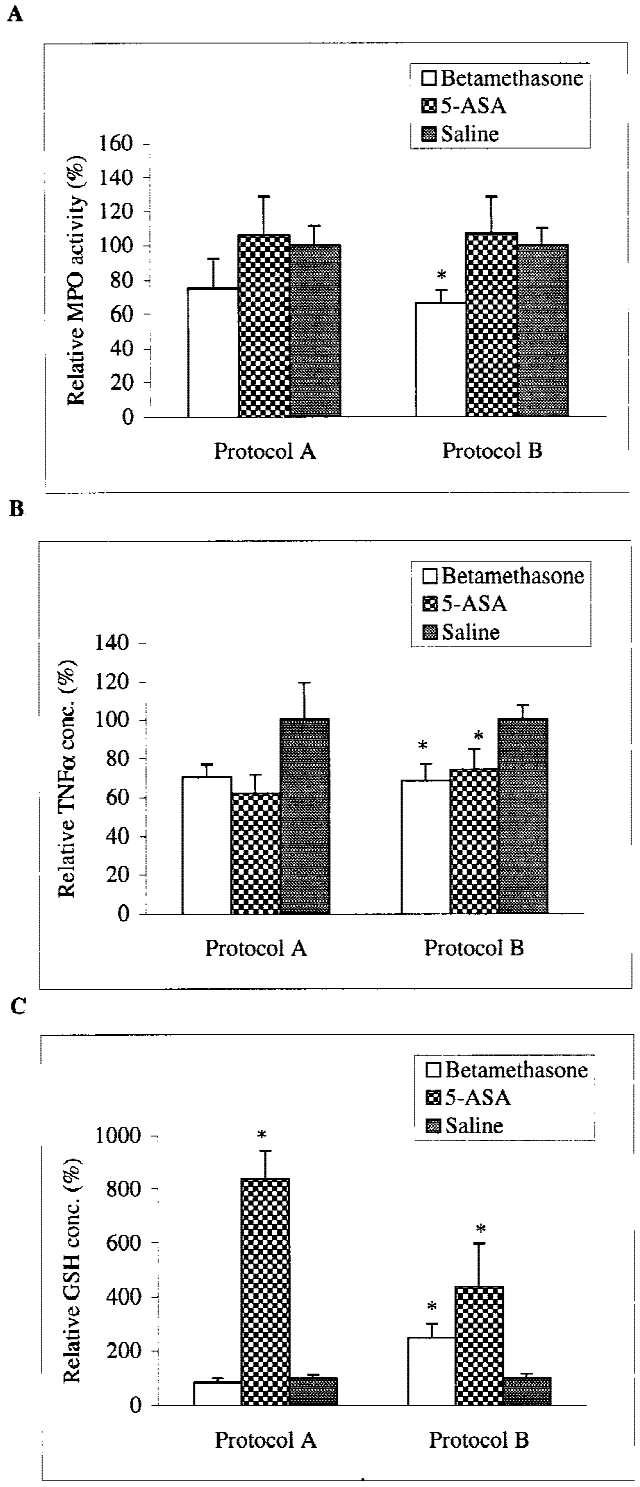

Fig. 5. The effect of 5-ASA or betamethasone on MPO activity (top), TNFα concentration (center) and GSH tissue levels (bottom) as measured in specimens taken from inflamed regions of the colon of DNBS induced rats after Treatment A and Treatment B. Results are expressed in percentage of saline controls. Shown are the mean values of at least 5 rats ± SEM. * $p < 0.05$ compared to saline control group.

in the colon of both patients and animal models induced with experimental colitis (18,19) have formed the basis for the hypothesis that antioxidant compounds can be used for the local treatment of colitis (3,20–23). In the present study, the therapeutic potential of the two antioxidant enzymes—SOD and catalase—was explored. SOD catalyzes the dismutation of the superoxide anion free radical to hydrogen peroxide and O_2. Catalase catalyzes the reduction of hydrogen peroxide to O_2 and water. After being produced in the inflamed epithelium of the colon by activated neutrophils, both superoxide anion and hydrogen peroxide can further interact with other compounds to create additional toxic materials, e.g. the creation of hypochlorous acid from chlorine and hydrogen peroxide. Our results suggest that enzymatic inactivation of the two oxidants can reduce the severity of colonic inflammation. This suggests that a combined, local treatment of catalase and SOD could be superior to the individual treatments described in this study. This assumption will be pursued in future studies.

In most studies in which antioxidants were used for colitis therapy, they were administered systematically either intravenously (20), intraperitoneously (3) or orally (22). A therapeutic response was observed in a phase II study in Crohn's disease patients when SOD was administered subcutaneously (4). The lack of studies involving local (intracolonic) treatment with antioxidant compounds is surprising in view of the large number of reports on the successful systemic treatments of the disease. A possible reason could be low efficacy, as was found by Keshavarzian and coworkers who report that while IP injection of WR-2721 (a glutathione-sparing compound) significantly decreased the severity of colitis in the rat, rectal administration of the compound did not (3). Such low efficacy could be a result of short residence time of the antioxidants in the vicinity of the colonic mucosa.

Cationization of proteins as a tool to increase their residence time in target organs has been suggested in the past (6). The ability of cationized proteins to adhere to negatively charged tissues poses an interesting potential in drug delivery. Local treatment of colonic inflammation with antioxidant enzymes is a rational example because of the accessibility of the injured mucosa to the lumen of the colon. The ultra-short half-life of the superoxide radical and hydrogen peroxide which are formed at the site of inflammation requires that antioxidant enzymes reside at that location as close as possible, to prevent the damage caused by the decomposition of these two ROS. For that purpose, the anionic side carboxyl groups of the enzymes were substituted with positively charged hexadiamine to allow the enzymes to adhere to the negatively charged mucosa. The adherence of the cationized enzymes to the colonic mucosa (Figure 1 and Figure 2) can be related to interaction with negatively charged proteoglycans like heparan sulphate, chondroitin sulphate and hyaluronic acid. Barbour and Hopwood (24) found that cationized ferittin attached rapidly to the tips of the colonic microvilli and even penetrated the cells by endocytosis. They also could adhere to the mucus lining of the colonic epithelium by reacting with negatively charged polysaccharides such as sialic acid. However, although our results demonstrate that cationized catalase and cationized SOD adhered to the colonic epithelium, it was difficult to point out the exact site of attachment which is now the subject of an ongoing study.

In this study we have demonstrated that cationized catalase and SOD were more efficient in their ability to protect the colonic tissues against inflammation. The cationized enzymes adhered to the colonic epithelium for at least one hour; an observation that verified the assumption that cationization was the cause for the adherence of the enzymes to the colonic

epithelium. In addition, the cationized enzymes were more effective than 5-ASA in the two colitis-induction protocols used and better than betamethasone in the prevention treatment (Treatment A) in moderating the induced inflammation. Still, the cationized enzymes were found to be superior to the native ones only in the inflamed regions of the rat colon, a finding which may indicate that ulcerated tissues are injured far beyond antioxidant enzyme therapy capabilities. It suggests that the cationized enzymes would not be able to adhere to ulcerated tissues as was observed in duodenal and gastric ulcers. The ulcerated tissues were found to contain high concentrations of positively charged proteins that increase the affinity to negatively charged substances, such as sucralfate (25). It is speculated that if similar changes in the electric charge occur in ulcerated regions of the colon, the ability of cationized antioxidant enzymes to adhere to ulcer regions will be limited.

Experimental colitis was induced in the rat by intracolonic administration of DNBS. Like TNBS, two types of tissue injuries macroscopically characterize the colitis induced by DNBS: ulceration and defined regions of inflammation. The distinction between the two was easily visualized, and in a related study the amount of inflammatory cells and tissue MPO activity and TNFα concentration were significantly higher in the ulcerated tissues than in the inflamed tissue (unpublished data). MPO activity and TNFα tissue concentration was different after Treatment A and Treatment B. While the latter caused a reduction in both MPO activity and TNFα concentrations, the former caused a significant reduction in tissue MPO activity but did not affect TNFα concentrations (Figure 3, Figure 4).

MPO, an enzyme found predominantly in activated neutrophils, is commonly used to quantify acute colonic inflammation (12). Still, MPO activity cannot be used to monitor macrophage- or lymphocyte-mediated inflammation. The latter cells accumulate in the inflamed colon at later stages of the inflammatory process. Accumulation of neutrophils in colonic tissues within one day after colitis induction by DNBS was reported by Hawkins and coworkers (26). In their study macrophages and lymphocytes were observed in all layers of colonic tissues a few days after colitis induction. A similar phenomenon was observed histologically in our laboratory in specimens taken from the colon of DNBS induced rats (data not shown).

TNFα, a mediator released mainly from activated macrophages and T-lymphocytes, has a crucial role in the pathophysiology of intestinal inflammation in IBD patients and experimentally induced animal models (27,28). The short duration of inflammation of Treatment A was not enough to cause an increase in TNFα concentrations. The cytokine levels were similar in the naive control animals and in the study groups (data not shown). In Treatment B, however, TNFα levels decreased. This could be explained by either of the following mechanisms: (a) the production of TNFα was inhibited by the cationized enzymes, or (b) the accumulation of TNFα secretory cells was inhibited by the enzymes. The former assumption is supported by Reimund and co-workers (29) who found an *in vitro* reduction in TNFα production in tissue specimens taken from IBD patients after treatment with several antioxidant compounds.

Among the various endogenous antioxidant defense systems, GSH is considered to be the most important one. A depletion of GSH levels in IBD patients and experimental colitis rat model was previously reported (18,30). However, the relation between colonic inflammation as measured by MPO activity and tissue GSH concentration is controversial. For example, Cruz and coworkers reported on reduction of MPO activity which was associated with reduction in GSH depletion following treatment of TNBS-induced rats with an antioxidant flavonoid (22). On the other hand, the basal GSH levels were preserved with no reduction in MPO activity after similar flavonoid treatment in acetic acid induced colitis in rats (21). In the present study, a discrepancy was found between the changes in MPO activity and GSH concentration. This observation suggests that the anti-inflammatory properties of the cationized antioxidant enzymes are unrelated to their glutathione spearing properties.

The clinical implication of using cationized antioxidant enzymes for the treatment of IBD was estimated, in our study, by comparing their efficacy to that of local administration of 5-ASA and betamethasone (Figure 5). In general, the anti-inflammatory effect of the cationized antioxidant enzymes was found to be superior to the two commercial products. Our results are partially supported by previous reports, which indicated that 5-ASA and betamethasone were ineffective when administered intracolonically to pre-colitis inducted rats (31). However, they were found to be effective when administered post-colitis induction (32). The high GSH levels observed after the treatment with 5-ASA (Figure 5C) suggest that 5-ASA acts more as a scavenger preventing GSH depletion than an anti-inflammatory agent.

In conclusion, cationized antioxidant enzymes are effective tools for the local treatment of colitis. In the experimental colitis model used, the cationized enzymes were found to be more effective than both the native enzymes and the commonly used drugs 5-ASA and betamethasone. This study clearly demonstrates that the attachment of antioxidant enzymes is a feasible method to improve the anti-inflammatory effect of antioxidant enzyme therapy.

ACKNOWLEDGMENTS

The study was supported by a research grant from the American-Israeli Binational Foundation number 93-56. The results reported here are included in the dissertation thesis of S.B. as partial fulfilment of her Ph.D. degree requirements at The Hebrew University of Jerusalem. The helpful discussions, comments, and good will of Professor Miles Epstein, Department of Anatomy, University of Wisconsin Medical School and the skillful technical assistance of Ms. Naama Levin are greatly acknowledged.

REFERENCES

1. M. B. Grisham and D. N. Granger. Neutrophil-mediated mucosal injury. Role of reactive oxygen metabolites. *Dig. Dis. Sci.* **33**:6s–15s (1988).
2. C. F. Babbs. Oxygen radicals in ulcerative colitis. *Free Radic. Biol. Med.* **13**:169–181 (1992).
3. A. Keshavarzian, J. Haydek, R. Zabihi, M. Doria, M. D'Astice, and J. R. Sorenson. Agents capable of eliminating reactive oxygen species. Catalase, WR-2721, or Cu(II)2(3,5-DIPS)4 decrease experimental colitis. *Dig. Dis. Sci.* **37**:1866–1873 (1992).
4. J. Emerit, S. Pelletier, J. Likforman, C. Pasquier, and A. Thuillier. Phase II trial of copper zinc superoxide dismutase (CuZn SOD) in the treatment of Crohn's disease. *Free Radic. Res. Commun.* **2**:563–569 (1991).
5. R. Kohen and R. Shalhoub. Prevention and induction of oxida-

tive damage in E. coli cells by cationized proteins. *Free Rad. Biol. Med.* **16**:571–580 (1994).
6. J. Schalkwijk, W. B. Van den Berg, L. B. Van de Putte, L. A. Joosten, and L. Van den Bersselaar. Cationization of catalase, peroxidase, and superoxide dismutase. Effect of improved intra-articular retention on experimental arthritis in mice. *J. Clin. Invest.* **76**:198–205 (1985).
7. R. Kohen, A. Kakunda, and A. Rubinstein. The role of cationized catalase and cationized glucose oxidase in mucosal oxidative damage induced in the rat jejunum. *J. Biol. Chem.* **267**:21349–21354 (1992).
8. A. Rubinstein, A. Kakunda, and R. Kohen. Protection of the rat jejunal mucosa against oxidative injury by cationized superoxide dismutase. *J. Pharm. Sci.* **82**:1285–1287 (1993).
9. H. Aebi. Catalase *in vitro. Methods Enzymol.* **105**:121–126 (1984).
10. J. M. McCord and I. Fridovich. Superoxide dismutase. An enzymatic function for erythrocuprein. *J. Biol. Chem.* **244**:6049–6055 (1969).
11. J. L. Wallace, T. Le, L. Carter, C. B. Appleyard, and P. L. Beck. Hapten-induced chronic colitis in the rat: Alternatives to trinitrobenzene sulfonic acid. *J. Pharmacol. Toxicol. Methods* **33**:237–239 (1995).
12. M. B. Grisham, J. N. Benoit, and D. N. Granger. Assessment of leukocyte involvement during ischemia and reperfusion of intestine. *Methods Enzymol.* **186**:729–733 (1986).
13. R. Paroni, E. De Vecchi, G. Cighetti, C. Arcelloni, I. Fermo, A. Grossi, and P. Bonini. HPLC with o-phthalaldehyde precolumn derivatization to measure total, oxidized, and protein-bound glutathione in blood, plasma, and tissue. *Clin. Chem.* **41**:448–454 (1995).
14. M. M. Bradford. A refined and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
15. J. H. Zar (ed.). *Biostatistical Analysis,* (2nd Ed.), Prentice-Hall, Englewood Cliffs, NJ, 1984.
16. M. L. Harris, H. J. Schiller, P. M. Reilly, M. Donowits, M. B. Grisham, and G. B. Bulkley. Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: Cause, consequence or epiphenomenon? *Pharmacol. Ther.* **53**:375–408 (1992).
17. M. B. Grisham. Oxidants and free radicals in inflammatory bowel disease. *Lancet* **344**:859–861 (1994).
18. G. D. Buffinton and W. F. Doe. Depleted mucosal antioxidant defences in inflammatory bowel disease. *Free Radic Biol. Med.* **19**:911–918 (1995).
19. S. Blau, R. Kohen, P. Bass, and A. Rubinstein. Changes in total low molecular weight antioxidant profiles at different severities of colonic mucosa inflammation in the dinitrobenzen sulfonic acid induced rat model. *Dig. Dis. Sci.* In press.
20. F. N. Koksoy, H. Kose, G. R. Soybir, O. Yalcin, and B. Cokneseli. The prophylactic effects of superoxide dismutase, catalase, desferrioxamine, verapamil and disulfiram in experimental colitis. *J. R. Coll. Surg. Edinb.* **42**:27–30 (1997).
21. J. Galvez, T. Cruz, E. Crespo, M. A. Ocete, M. D. Lorente, F. Sanchez de Medina, and A. Zarzuelo. Rutoside as mucosal protective in acetic acid-induced rat colitis. *Planta Med.* **63**:409–414 (1997).
22. T. Cruz, J. Galvez, M. A. Ocete, M. E. Crespo, L. H. F. Sanchez de Medina, and A. Zarzuelo. Oral administration of rutoside can ameliorate inflammatory bowel disease in rats. *Life Sci.* **62**:687–695 (1998).
23. T. Yoshikawa, T. Yamaguchi, N. Yoshida, H. Yamamoto, S. Kitazumi, S. Takahashi, Y. Naito, and M. Kondo. Effect of Z-103 on TNB-induced colitis in rats. *Digestion* **58**:464–468 (1997).
24. W. M. Barbour and D. Hopwood. Uptake of cationized ferritin by colonic epithelium. *J. Pathol.* **139**:167–178 (1983).
25. R. Nagashima. Mechanisms of action of sucralfate. *J. Clin. Gastroenterol.* **3**:117–127 (1981).
26. J. V. Hawkins, E. L. Emmel, J. J. Feuer, M. A. Nedelman, C. J. Harvey, H. J. Klein, H. Rozmiarek, A. R. Kennedy, G. R. Lichtenstein, and P. C. Billings. Protease activity in a hapten-induced model of ulcerative colitis in rats. *Dig. Dis. Sci.* **42**:1969–1980 (1997).
27. H. Takizawa, N. Shintani, M. Natsui, T. Sasakawa, H. Nakakubo, T. Nakajima, and H. Asakura. Activated immunocompetent cells in rat colitis mucosa induced by dextran sulfate sodium and not complete but partial suppression of colitis by FK506. *Digestion* **56**:259–264 (1995).
28. H. M. van Dullemen, S. J. van Deventer, D. W. Hommes, H. A. Bijl, J. Jansen, G. N. Tytgat, and J. Woody. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* **109**:129–135 (1995).
29. J. M. Reimund, A. C. Allison, C. D. Muller, S. Dumont, J. S. Kenney, R. Baumann, B. Duclos, and P. Poindron. Antioxidants inhibit the *in vitro* production of inflammatory cytokines in Crohn's disease and ulcerative colitis. *Eur. J. Clin. Invest.* **28**:145–150 (1998).
30. F. Sanchez de Medina, J. Galvez, J. A. Romero, and A. Zarzuelo. Effect of quercitrin on acute and chronic experimental colitis in the rat. *J. Pharmacol. Exp. Ther.* **278**:771–779 (1996).
31. R. N. Fedorak, L. R. Empey, C. MacArthur, and L. D. Jewell. Misoprostol provides a colonic mucosal protective effect during acetic acid-induced colitis in rats. *Gastroenterology* **98**:615–625 (1990).
32. K. J. Torsher, L. R. Empey, and R. N. Fedorak. Misoprostol therapy following trinitrobenzene sulfonic acid-induced colitis accelerates healing. *Prostaglandins Leukot. Essent. Fatty Acids* **45**: 275–281 (1992).