Acute colitis induction by oil of mustard results in later development of an IBS-like accelerated upper GI transit in mice

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Oil of mustard (OM), allyl isothiocyanate, is one of a number of small molecular weight compounds that constitute the pungent components of mustard, horseradish, and wasabi. It is a direct stimulant of small nerve fibers (2), a potent acute inflammatory irritant (14), and has been used experimentally to evoke visceral hyperalgesia following intracolonic administration (15). As a neuronal stimulant, it has been used to successfully study allodynia and visceral hyperalgesia in mice (19, 29) and to elucidate the sensory neural pathways activated in response to its application (3, 20, 26). In the GI tract, intracolonic application of a 0.5% solution of OM rapidly induces visceral hyperalgesia that can be measured as heightened sensitivity to von Frey hairs applied to the abdomen (19) and during graded changes in intraluminal pressure via an indwelling balloon catheter (29). Altered sensitivity to these stimuli occurs within minutes after OM administration. Although a transient influx of neutrophils and eosinophils that peaked at 4 h has been observed (26), the effects of OM as an inflammatory irritant in the GI tract have not been studied beyond 8 h. Our aim was to characterize and investigate the effects of OM as a model of acute colitis in mice. In addition, we hypothesized that this could induce altered gut motility at time points well beyond resolution of and recovery from the initial inflammatory insult and thus provide a potential model for postinflammatory IBS (PI-IBS).

Thus we report here that intracolonic application of a 0.5% solution of OM produced a severe, transient colitis characterized by body weight loss, colon shrinkage, edema and thickening, severe diarrhea, and transmural inflammatory lesions that occasionally penetrated through the seromuscular layers. Colitis was maximum at day 3 and gradually diminished in intensity with less severe symptoms by day 7 followed by complete resolution and recovery by day 14. When examined for long-term changes in GI function, OM-treated mice had significantly accelerated upper GI transit rates measured on days 21 and 28 post-OM application compared with age-matched untreated controls. Mice 28 days post-OM demon-

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stratified increased sensitivity of upper GI transit to inhibition by the \( \mu \)-opioid receptor agonist loperamide compared with control mice. Our results suggest that OM-induced colitis and OM-induced accelerated upper GI transit in mice can be used as a new experimental model of functional bowel disorders associated with PI-IBS.

**MATERIALS AND METHODS**

**Mice.** Male CD-1 mice (Charles River Laboratories, Kingston, NC), 9–10 wk old, were used throughout these studies. All treatments were carried out in accordance with the Federal Animal Welfare Act and with methods approved by the Institutional Animal Care and Use Committee of Johnson and Johnson Pharmaceutical Research and Development.

**Materials.** Freshly opened OM (95 or 98% pure allyl isothiocyanate; Sigma-Aldrich St. Louis, MO) was used in each experiment. Carmine dye (cochineal powder; CI 75470) was purchased from Sigma-Aldrich. For immunohistochemical studies, rabbit polyclonal antibody to myeloperoxidase was obtained from DAKO Cytomation (Carpinteria, CA). A rat monoclonal antibody to mouse F4/80 (Clone A3–1) was obtained from Abcam (Boston, MA), and rat monoclonal antibody to mouse CD45 (leukocyte common antigen; Clone 30-F11) was obtained from Pharmingen (San Diego, CA). Biotinylated goat anti-rabbit and biotinylated goat anti-rat secondary antibodies, and avidin-horseradish-peroxidase biotin were obtained from Vector Laboratories (Burlingame, CA). 3,3’-Diaminobenzidine was purchased from Biomedica (Foster City, CA).

**Induction of colitis.** Mice were briefly anesthetized with ketamine/xylasine (Sigma) and held in a vertical position so that 50 \( \mu l \) of a solution of 0.5% OM in 30% ethanol could be administered intracolonically. The OM administration occurred to a depth of 4 cm via a syringe equipped with a ball-tipped 22-G needle.

Body weights were measured daily. At the termination of experiments, colons were resected, examined for signs of inflammation, weighed after removing fecal contents (which were examined for signs of diarrhea), and the length from the aboral end of the cecum to the anus was determined. These data and observations were assigned a score as indicated in Table 1. The sum of the individual macroscopic indexes was combined into a macroscopic score for each colon, where 0 is normal and 15 is maximally affected.

**Histology evaluations.** Segments from the distal colon taken from the first to fourth centimeter from the anus of each animal were removed, rinsed in saline, fixed in 10% neutral buffered formalin, embedded longitudinally in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The tissues were examined for epithelial damage using light microscopy by an investigator who was blinded to the sample groups and were then scored for epithelial damage, cellular infiltration, and damage or alteration of smooth muscle architecture by a second investigator according to the scale detailed in Table 2. The sum of these individual histology scores comprised a microscopic score for each colon, where 0 is normal and 9 is maximally affected.

**Immunohistochemistry.** Immunohistochemical (IHC) methods were applied to characterize the inflammatory cells in the paraffin-embedded tissues according to previously described methods (9). Briefly, 5-\( \mu m \) tissue sections were mounted on microscope slides and then routinely dewaxed and rehydrated. After a 5-min exposure in a microwave in target buffer (DAKO) and subsequent treatment in 3% \( \text{H}_2\text{O}_2 \) for 5 min, the slides were routinely processed for IHC. All incubations were performed at room temperature for 30 min. After a 10-min blocking step with normal goat serum, the tissues were incubated with the primary antibodies. Rabbit anti-myeloperoxidase was used to identify neutrophils, rat anti-mouse F4/80 to detect macrophages, and rat anti-mouse CD45 to detect lymphocytes, predominately T cells, in the infiltrated cell populations. After brief

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<thead>
<tr>
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<td>diarrhea severe inflammation and/or extensively distributed</td>
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<td>1–2 focal areas or extent of inflammatory infiltrate ≤ 33% of tissue length</td>
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Separate index scores for macroscopic parameters were determined for each mouse. A macroscopic score was calculated as the sum of the index scores for each mouse. Epithelial damage was assessed as blunted mucosa, destruction of crypt architecture, and/or loss of epithelium. Muscle damage was assessed as submucosal edema, hyperplasia, and/or loss of architecture.
OM induces body weight loss and severe colitis. After intracolonic instillation of 0.5% OM, body weights were measured daily. The mice rapidly lost weight during the first three days post-OM, with maximum mean body weight losses as high as 11% by day 3. The mice then gradually regained their weight until by day 7 their weights were indistinguishable from those of untreated mice (Fig. 1). Mice treated with ethanol vehicle did not show acute weight losses.

Because the most severe body weight loss occurred at day 3, we examined the tissues of mice at that time for evidence of colitis. Distal colons from OM-treated mice were shortened and thickened compared with colons from untreated mice and from mice given only 30% ethanol vehicle, and exhibited bloody lesions (Fig. 2). The distal colon and portions of the proximal colon were more distended than those of normal mice. Proximal colons from many other mice studied were highly distended and contained thick mucus or watery diarrhea.

Data shown in Table 3 reveal that at 3 days post-OM, the distal colons were shorter (by 2.4 ± 0.5 cm; \( P < 0.05 \)) and had a greater mass (by 0.126 ± 0.070 g; \( P < 0.05 \)) compared with their untreated controls. The change in length represented a 22% decrease, and the change in colon weight represented a 36% gain compared with untreated controls. The mice exhibited diarrhea scores of 2.2 ± 0.5 (\( P < 0.05 \) vs. untreated). Colon damage scores for OM-treated mice were 3.0 ± 0.6 (\( P < 0.05 \) vs. untreated), with a number of mice exhibiting bloody penetrating lesions in the distal colon at sites that did not correspond to the site where OM was applied. Values obtained for ethanol-treated mice were unchanged compared with untreated mice.

The proximal colon tissues exhibited a generalized mild erythema and thickening, but no overt microscopic lesions were observed.

Histological evaluation of tissues from OM-treated mice. Tissues collected at day 3 for H&E staining revealed discontinuous lesions with loss of epithelial architecture, penetration through the submucosa, and loss of smooth muscle architecture.

Table 3. Colitis parameters 3 days post-oil of mustard application

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated Mice</th>
<th>EIOH treated</th>
<th>0.5% Oil of Mustard</th>
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<td>Colon length, cm</td>
<td>10.9 ± 0.2</td>
<td>10.9 ± 0.2</td>
<td>8.5 ± 0.6*</td>
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<tr>
<td>Colon weight, g</td>
<td>0.349 ± 0.051</td>
<td>0.321 ± 0.012</td>
<td>0.474 ± 0.071*</td>
</tr>
<tr>
<td>Stool score</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2.2 ± 0.5*</td>
</tr>
<tr>
<td>Colon damage score</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>3.0 ± 0.6*</td>
</tr>
<tr>
<td>Microscopic score</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>9.0 ± 0.6*</td>
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Values are means ± SE from a single experiment (n = 9 per group). 0.5% oil of mustard in 30% ethanol, or 30% ethanol alone were applied intracolonically to CD-1 mice. After 3 days colons were examined for damage. Macroscopic and microscopic scores are as described in MATERIALS AND METHODS. One of two experiments yielding identical results. \(*P < 0.05\) vs. untreated mice, ANOVA.
There was little or no histological damage observed in tissues treated only with the 30% ethanol vehicle.

In tissues from OM-treated mice, a cellular infiltrate was observed that was most intense in regions of epithelial and smooth muscle damage but was also noticeable in the submucosa and muscularis mucosa of areas that did not exhibit overt pathology in the mucosa or muscularis externa. Immunohistochemical staining of the cellular infiltrates in the lesioned sections from OM-treated mice (Fig. 4) show the epithelial, submucosal, and smooth muscle regions invaded by neutrophils (myeloperoxidase positive) and lymphocytes (leukocyte common antigen; CD45 positive). Macrophages (F4/80 positive) were concentrated in the smooth muscle below these lesioned areas. In other areas of the distal colon near a lesioned area, but where the epithelium was intact, the smooth muscle was hyperplastic. Lymphocytes could be observed in the epithelium but were especially dense in the submucosa and mucosa muscularis as well as in the smooth muscle layer. Neutrophils were essentially found in high density in the submucosa, whereas F4/80+ macrophages were evident in the epithelial layer and were highly prominent in the submucosa and smooth muscle layer. No recruited cells and no immunohistochemical staining for lymphocytes, macrophages, or neutrophils was observed in the smooth muscle or submucosa in tissues taken from mice treated with the 30% ethanol vehicle. Epithelium in these tissues exhibited some LCA+ lymphocytes and F4/80+ stained macrophages, but no MPO+ neutrophils. These data demonstrate that the inflammatory response
scores for untreated mice were 0.05 vs. untreated mice. Only mice given OM showed significant damage vs. untreated), and restored to normal by untreated), were reduced at means, whereas mice given EtOH had no significant damage, and damage scores for untreated mice were 0. Only mice given OM showed significant damage scores in animals that were examined for symptoms of colitis (data not shown).

Fig. 5. Time course of OM-induced colitis. Groups of mice were evaluated at day 3, 7, or 14 after intracolonic administration of 0.5% OM. Data shown are means ± SE for macroscopic scores and for microscopic scores (n = 6). *P < 0.05 vs. untreated mice. Only mice given OM showed significant damage scores, whereas mice given EtOH had no significant damage, and damage scores for untreated mice were 0.

is engendered by OM as a fully integrated one composed of apparently large numbers of lymphocytes, macrophages, and neutrophils.

H&E-stained tissues from proximal colons revealed edema and swelling of the muscularis externa, although no prominent cellular infiltrate was observed in this region of the bowel (data not shown).

Time course of OM-induced colitis. The colitis induced by OM did not extend beyond 7 days. The macroscopic damage scores in animals that were examined for symptoms of colitis were greatest on day 3 (8.8 ± 1.4, n = 5, P < 0.05 vs. untreated), were reduced at day 7 (3.4 ± 0.7; n = 5, P < 0.05 vs. untreated), and restored to normal by day 14 (Fig. 5). The mean histology score on day 3 was 4.8 ± 1.7 (P < 0.05 vs. untreated), declined by day 7 to 2.2 ± 0.6 (P < 0.05 vs. untreated), and declined further by day 14 to 1.0 ± 0.6 (P = NS vs. untreated). Ethanol essentially had no effect on macroscopic or microscopic scoring. These data therefore demonstrate that intracolonic OM produces an acute, transient, and severe tissue response in the distal colon. The response at day 3 appears to have many of the hallmarks of colitis but resolves within days of its induction.

OM induces a delayed postinflammatory accelerated upper GI transit. Our original objective was to determine whether intracolonic OM could result in altered GI motility at a point in time that was distant from the original insult. We therefore examined upper GI transit at times when there was no longer any evidence of inflammation, starting with day 14, and extending to days 21 and 28. Upper GI transit was increased at days 21 and 28 post-OM administration compared with untreated controls (Fig. 6). In these studies, upper GI transit increased significantly (P < 0.05) from a mean of 58 ± 1% in untreated controls to a mean of 73 ± 2% at day 28 post-OM. Upper GI transit was slightly less at day 21 (70 ± 2%) vs. day 28 but was still significantly higher compared with transit in untreated mice (P < 0.05). In mice that had been given only the ethanol vehicle 28 days before, we found that upper GI transit was no different than that seen in untreated mice. These results clearly demonstrate that intracolonic OM reproducibly induces accelerated GI transit in the small intestine, a site far removed from the fourth centimeter of the mid-distal colon where OM application occurred and at a time several weeks following the acute inflammatory response. This accelerated upper GI transit developed gradually and suggests that progressive tissue or receptor remodeling may have occurred as a consequence of OM-induced inflammation.

Inhibition of accelerated upper GI transit by loperamide. GI inflammation in mice induced by oral administration of croton oil has previously been reported to increase GI transit rates and to increase sensitivity to inhibition by opiates (32, 33). We thought that the post-OM increase in GI transit might serve as a PI-IBS model, and to further explore this premise, we determined the activity of loperamide, a peripherally active μ-opioid receptor agonist, in this model. Results show that (Fig. 7) loperamide significantly inhibited transit at the lowest dose tested (0.1 mg/kg) in the day 28 post-OM group (P < 0.001 vs. OM control, P < 0.05 vs. loperamide-treated normal mice), but not in the untreated mice, and exhibited a shift toward greater inhibition at higher doses compared with normal mice. These results demonstrate altered gut motility in OM-treated mice that is associated with increased sensitivity to a μ-opioid receptor agonist.
Normal age-matched mice were similarly treated with loperamide or vehicle. were examined for upper GI transit and compared against vehicle-treated mice. Twenty-eight days later, loperamide was administered intragastrically by required. This further strengthens the concept that OM-induced chemokines and upregulation of cell adhesion molecules is cellular compartment. For an immune response such as this to a fully integrated immune response and not confined to a single response induced by OM is composed of lymphocytes, macromet. These data demonstrate that the inflammatory muscle thickening, but where there was no evidence of epithelial damage. The epithelium and smooth muscle in these sections also contained lymphocytes and macrophages but no neutrophils. These data demonstrate that the inflammatory response induced by OM is composed of lymphocytes, macromet., and neutrophils in high proportions, consistent with a fully integrated immune response and not confined to a single cellular compartment. For an immune response such as this to have occurred, a complex signaling cascade of cytokines and chemokines and upregulation of cell adhesion molecules is required. This further strengthens the concept that OM-induced colitis, even if acute and rapidly resolved, might be an adequate model for human clinical disease. Although the overt and microscopic tissue damage occurred principally in the distal colon, there was edema in the proximal colon, but no significant inflammatory lesions were found there.

Fig. 7. OM increases sensitivity to μ-opioid inhibition of upper GI transit. Groups of mice (n = 12–14) were administered 0.5% OM intracolonically. Twenty-eight days later, loperamide was administered intragastrically by gavage 1 h before carmine dye delivery by gavage. Twenty minutes later, mice were examined for upper GI transit and compared against vehicle-treated mice. Normal age-matched mice were similarly treated with loperamide or vehicle. *P < 0.05, loperamide vs. OM-treated mice (vehicle treated). **P < 0.05, loperamide vs. normal mice (vehicle treated). †P < 0.05, OM-treated mice vs. corresponding loperamide dose in normal mice.

OM is known to be proinflammatory, but that activity extends mainly to its ability to induce plasma extravasation, which is principally due to the release of substance P and to neurokinin 1 receptor (NK1R) activation (1, 14, 15, 24). OM is known to signal via NK1R by studies showing diminished plasma extravasation after NK1R antagonist treatment. However, NK1R activation is an early event because its antagonism was effective only in the 15 min immediately following OM application (14). Plasma extravasation still occurred in NK1R knockout mice and in denervated mice, suggesting a nonneuronal component to the mechanism activated by OM (20, 40). Plasma extravasation in this model was also not dependent on mast cells, because OM-induced changes did not occur in mast cell-deficient mice, and the mast cell-derived products serotonin and histamine do not appear to play a role in OM skin inflammation (14). OM-induced plasma extravasation was not inhibited by antagonism of CGRP (24) or by L-NAME inhibition of NO synthase (14).

There are earlier reports suggestive of direct tissue damage by OM (10, 44). In one study (26), within hours of intracolonic administration, OM was shown to induce a cellular infiltrate that was primarily composed of neutrophils and eosinophils. However, that infiltrate diminished between 4 and 8 h, and there were no further examinations for effects on tissues at later time points as were performed in the present study. In other investigations, topical OM application to mouse ears was reported to cause tissue damage but only by OM concentrations >5% (14), whereas the present studies were conducted with 0.5% OM. Therefore, it is not clear whether the damage observed in the present study was exclusively neurally initiated or was via direct effects on tissue or was due to both.

The time course of the colitis we observed was unique compared with that seen in other models of colitis. OM-induced colitis peaked at 3 days and then resolved between days 7 and 14 postapplication. In comparison, trinitrobenzene sulfonic acid (TNBS)-induced colitis continues to develop past day 3 and increases in severity through days 7 to 14, after which there is resolution of damage. Lesions in TNBS colitis are continuous, although the severity is similar to that seen in OM colitis. Dextran sulfate sodium (DSS)-induced colitis does not begin to manifest symptoms until 4 or 5 days have elapsed and is dependent on daily DSS intake. Overt tissue damage in DSS colitis is discontinuous but not as severe as that seen in OM colitis.

The second important result originating from our studies with intracolonic OM is the demonstration that there were functional bowel changes that were spatially and temporally removed from the induction of colitis and when inflammation could no longer be detected. Thus upper GI transit was significantly accelerated compared with transit seen in untreated mice. Oral administration of croton oil or castor oil, which are proinflammatory substances, induce an immediate accelerated upper GI transit up to 4 days later (35). It is therefore noteworthy that the accelerated small bowel transit induced by a single intracolonic OM administration was observable at days 21 and 28. Because it was our intention to explore postinflammatory changes, we did not pursue functional bowel changes at earlier time points when there was active inflammation. This is an animal model in which postinflammatory functional bowel changes manifested as acceler-
ated upper GI transit develop weeks after the inflammatory episode was initiated. In addition, accelerated upper GI transit was dose responsively reduced by the μ-opioid agonist loperamide, with greater sensitivity to the compound than seen when administered to normal mice. This is reminiscent of, and consistent with, results reported for opiate-mediated correction of upper GI transit dysfunction following oral croton oil administration and had been considered to be a reflection of increased opiate receptor expression (32, 35). This finding is also consistent with other experimental models of inflammation-induced gut dysfunction that have been characterized by persistent changes in neuronal and smooth muscle function several weeks after recovery from an initial inflammatory insult to the gut epithelium (6, 7, 41, 43), but these functional changes followed a more chronic intestinal infection. Long-term changes included impaired neurotransmitter release from enteric nerve endings (7, 41) and enhanced neuromuscular contractile activity (43).

OM is a neuronal stimulant (2, 10, 16, 17, 45, 46). As such, it has been used to great effect to study neuronal signaling in transduction of pain in peripheral tissues including viscera (4, 13, 19, 29, 40, 42). Intracolonic OM is reported to induce hyperalgesic responses to intracolonic balloon distension and also hyperalgesic responses to abdominally applied von Frey hairs (19, 29). The latter is an example of referred pain, and signifies neuronal signaling from visceral sites distant from the somatic structures that are stimulated but which share the same spinal nerve communication to the central nervous system. The visceral hyperalgesia in those reports occurred within 30 min of OM administration, as did the referred anterior abdominal wall hyperalgesia. The referred pain occurred at a site that was distant from the OM application site. Our observation of accelerated upper GI transit is similar, in that the OM application to the mid-distal colon resulted in alterations in gut function in the small intestine. There is no direct evidence yet to demonstrate that the effects by OM that we observed are due to neuronal activation, however.

The fact that we observed accelerated small-bowel transit consistently only after a 3- to 4-wk delay implies that gradual receptor remodeling, or tissue remodeling with receptor redistribution, might have occurred within the enteric nervous system and/or within visceral afferent pathways. There is precedent for neuroplastic alterations for NMDA receptor expression on nociceptive neurons as a consequence of OM stimulation, but these were short-term studies (4, 30). Various neuronal receptors, among them cannabinoid, NMDA, NK1, P2X, GABA, and μ-opioid, and soluble mediators that include nitric oxide and substance P, have been identified as being upregulated as a consequence of OM treatment (3, 4, 13–15, 23–26, 42, 46). Altered neuronal function in IBD includes morphological remodeling of intrinsic nerves expressing receptors for nerve growth factor essential to regrowth of nerves and repair of tissue damage but also overexpression of nerve growth factor receptor on fibroblasts, smooth muscle, and epithelial cells in response to inflammatory cytokines (34, 36, 39). Nerve growth factor regulates the expression of sensory neuropeptides (i.e., tachykinins and CGRP) (39) and expression of vanilloid VR1 receptor and purinergic P2X3 receptor, which have been implicated in postinflammatory hyperalgesia (34, 36). The signaling pursuant to OM application leading to colitis is not clearly understood yet, and the receptors or cell types that might be altered to change intestinal motility in this context have not yet been identified. In terms of motility changes, it may therefore be instructive to note studies showing that soon after intracolonic TNBS administration to mice, there were alterations in enteric Type II AH neuron activity and excitability in the ileum and increases in amounts of serotonin secreted by enterochromaffin cells in that region of the bowel (21, 22). Data demonstrating altered expression for serotonin receptors and serotonin reuptake transporter have recently been obtained in tissues from patients with IBD and IBS (5, 11). Those data represent a postinflammatory outcome and thus support the idea that intracolonic OM may similarly induce remodeling in the upper GI tract. OM has recently been demonstrated to signal through a newly described member of the transient receptor potential family, ANKTM1 (18), and other studies reveal OM-induced signaling involves cannabinoid, opioid, GABA, and purinergic receptors (3, 13, 26, 42). Whether alterations in local concentrations of enteric neurotransmitters, receptors, or other factors are responsible for the post-OM-accelerated GI transit remains to be determined.

Taken together, the data in the present study suggest that intracolonic administration of OM to mice can be used to study short-term development of colitis, possibly through a neurogenic origin, and also could be applied to studying development of dysfunctional bowel syndromes at a postinflammatory point in time when there is no gross inflammation. Whereas it is attractive to hypothesize that OM-induced colitis and the subsequent IBS-like accelerated upper GI transit originate via neurogenic stimulation, there is as yet insufficient evidence to directly support this concept. Nonetheless, this model may have potential as an animal model for PI-IBS or other functional bowel disorders.

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REFERENCES


