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Effect of D-002 on the Pre-ulcerative Phase of Carrageenan-induced Colonic Ulceration in the Guinea-pig

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Abstract

D-002 is a natural mixture of higher aliphatic primary alcohols, isolated and purified from beeswax which has anti-inflammatory properties, reduces leukotrienes (LTB_4) and thromboxane B_2 (TXB_2) in exudated carrageenan-induced pleurisy, and has anti-ulcer activity in different experimental models. This study was conducted to determine the effect of D-002 on the pre-ulcerative phase of carrageenan-induced colonic ulceration in guinea-pigs.

Animals were randomly distributed among a negative control, a positive control group treated with the vehicle Tween 20 in H_2O and two experimental groups receiving D-002 at 25 and 50 mg kg^{-1}. All treated animals received degraded carrageenan for three days for induction of colonic ulceration. Significant reductions in wet weight, wall thickness, counts of infiltrating polymorphonuclear neutrophils and of macrophages, and histological index were observed in colonic mucosa of D-002-treated animals compared with controls.

It is concluded that D-002 has a protective effect on the pre-ulcerative phase of carrageenan-induced colonic ulceration in the guinea-pig.

D-002 is a natural mixture of higher aliphatic primary alcohols isolated and purified from beeswax; it contains 1-triacontanol (26·63%), 1-octacosanol (17·49%), 1-dotriacontanol (16·95%), 1-hexacosanol (15·34%) and 1-tetracosanol (13·24%). 1-Tetratriacontanol (2·24%) is present as a minor component.

D-002 is moderately anti-inflammatory and reduces leukotriene (LTB_4) levels in exudated carrageenan-induced pleurisy, has anti-ulcer activity in different experimental models (Carbajal et al 1995), and reduces thromboxane B_2 (TXB_2) in the gastric mucosa of rats with ethanol induced-ulceration (Carbajal et al 1996).

Inflammation and the release of mediators such as leukotrienes is a common occurrence in different pathological processes, including ulcerative diseases of the colon (Wallace et al 1992; Mascolo et al 1995). The ulcerative disease induced by carrageenan in guinea-pigs is a recognized experimental model that mimics such disturbances (Grasso et al 1973; Engster & Abraham 1976; Beeken 1988; Marcus et al 1989) and is therefore useful for evaluating the putative action of different drugs acting on this process.

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Taking these facts into account, this study was designed to investigate the effect of D-002 on the pre-ulcerative phase of carrageenan-induced colonic ulceration in guinea-pigs.

Materials and Methods

Animals

Male guinea-pigs, 200–250 g, from the National Center for Laboratory Animals Production (Cenpalab, Cuba), were housed in environmentally controlled rooms (25 ± 2 °C, 12-h light–dark cycle) with free access to standard chow (Cenpalab). They were randomly allocated to three experimental groups (n = 8 per group).

Degraded carrageenan

Carrageenan is a sulphated polygalactoside extracted from red algae (McGill et al 1977). It was obtained commercially from BDH Chemicals (Poole, UK) and partially hydrolysed as previously described (Ukabam et al 1983). In brief, carrageenan (15 g) was dissolved in distilled water (300 mL) at 60 °C; concentrated HCl (0·2 mL) was added and the solution was kept at 60 °C overnight, cooled to room temperature and neutralized with NaOH pellets. The filtered, hydrolysed carrageenan stock solution (50 g L^{-1}) was refrigerated until used.

Administration and dosage
D-002 was suspended in a 2% Tween 20 in H_2O (as vehicle). All treatment (1 mL kg^{-1}) was administered intraperitoneally for three days. The water used in the experiment was pyrogen-free. The experimental groups were: group 1, guinea-pigs treated with the vehicle Tween 20 in water only; group 2, guinea-pigs treated with D-002 (25 mg kg^{-1}); group 3, guinea-pigs treated with D-002 (50 mg kg^{-1}).

All guinea-pigs received degraded carrageenan aqueous solution (3%) as drinking fluid for three days. The average daily intake of carrageenan per animal was 5·8 g kg^{-1}.

A satellite fourth group of control animals (n = 2) received water only as drinking fluid.

Observations
Animals were observed twice-a-day; their physical condition and the characteristics of the faeces (collected in trays placed beneath the cages) were recorded. Body weights were measured the day before the initial treatment and every day during the experiment. At necropsy the large bowel was opened and carefully examined, after fixation, by use of a lamp with a magnifying attachment.

Microscopic examination
Tissue samples were taken from the proximal, middle and distal thirds of the caecum, the ascending colon, the mid-transverse colon, the descending colon and the rectum. They were fixed in a 10% buffer formaldehyde, embedded in paraffin, sectioned, and stained with haematoxylin and eosin. Sections were also stained with 2% toluidine blue to detect the presence of sulphated polysaccharide.

Assessment of wet weight
The wet weight of the caecum was assessed as described by Mascolo et al (1995). A 5-cm segment of caecum 3 cm proximal to the ascending colon was resected, the lumen was rinsed with ice-cold saline, and the segment was weighed. Results are expressed as the mean ± s.d. of results from eight samples.

As an index of tissue oedema, caecum wall thickness (mm) was measured in properly oriented haematoxylin-stained sections using a calibrated eye-piece reticule positioned perpendicular to the serosal and mucosal surfaces (Buell & Berin 1994).

To quantify infiltration by polymorphonuclear neutrophils (PMN) and by macrophages, cells were counted in each complete cross-section of mucosa as described by Nygard et al (1994). The mean PMN and macrophage counts were determined for three sections per animal in each group. The expressed mean was calculated from the mean observed for each animal and afterwards averaged for each group.

Assessment of histological damage
The 1-cm length of each histological section was divided into five fields and each field further divided into four equal subsections. Each subsection was histologically assessed by use of the score system: 1, < 5 inflammatory cells; 2, > 5 inflammatory cells + sulphated polysaccharide in the mucosa; 3, > 5 inflammatory cells + breach at the surface epithelium; and 4, > 5 inflammatory cells + breach at the surface epithelium + sulphated polysaccharide in the mucosa. Scores 1 and 2 were considered to be mild damage and scores 3 and 4 to reflect extensive and more severe damage. Each subsection was evaluated on a cumulative basis. The maximum score for each subsection was thus 16 and the total maximum score for each section was 80. The overall value of the scores for the five fields was taken as the histological index for that section. Mean values and standard deviations for the histological index were averaged for each group. All such measurements were made by an observer unaware of the treatment conditions of the animals.

Statistical analysis
Comparisons between groups of wet weight, wall thickness, and PMN and macrophage counts, and mean values of the histological index were performed by use of the non-parametric Mann-Whitney *U*-test. Comparison between groups of guinea-pigs with focal darker areas in the colonic mucosa (macroscopic observation) and of overall sum of scores for field and for treatment were performed using the Fisher exact probability test.

Results

Observations
No animal died during the study. No signs of toxicity were noted in any of the animals. On the last day of the experiment some animals produced pasty faeces without other alteration. This discrete alteration was found in both control (n = 5) and treated animals (n = 2). During the study the body weight was similar for all groups.

Macroscopic changes in the pre-ulcerative phase
When viewed by use of a lamp with a magnifying attachment the mucosae of the caecum in all animals of the control group (carrageenan only) showed focal areas darker than the surrounding

Table 1. Effect of administration of D-002 on the pre-ulcerative phase of carrageenan-induced colonic ulceration in guinea-pigs as measured by the wet weight of 5-cm caecum segments and by wall thickness.

Group	Dose ($mg\,kg^{-1}$)	Wet weight (g)	Wall thickness (μm)
Control	–	1·67 ± 2·6	151·87 ± 2·9
D-002	25	0·56 ± 3·1*	110·12 ± 9·5*
D-002	50	0·51 ± 2·5*	104·50 ± 4·0*

Results are means ± s.d. of results from eight animals. * $P < 0·0001$, significantly different from control (Mann-Whitney U-test).

Table 2. Effect of administration of D-002 on the pre-ulcerative phase of carrageenan-induced colonic ulceration in guinea-pigs as measured by infiltration of polymorphonuclear neutrophils and macrophages into the caecum.

Group	Dose ($mg\,kg^{-1}$)	Polymorphonuclear neutrophils	Macrophages
Control	–	20·71 ± 6·0	25·38 ± 2·1
D-002	25	11·42 ± 4·0*	14·82 ± 3·8**
D-002	50	10·10 ± 3·5*	12·41 ± 3·3**

Results are means ± s.d. of results from eight guinea-pigs. * $P < 0·001$, ** $P < 0·0001$, significantly different from control (Mann-Whitney U-test).

mucosa. These areas were rounded, oval or more elongated in shape and measured up to 0·5 mm in diameter and up to 1 mm in length; they numbered from five to fourteen. No macroscopic changes were observed in the remainder of the large bowel. In the group treated with D-002 at $25\,mg\,kg^{-1}$, macroscopic focal dark lesions similar to those of the control group were observed for two animals; in the group receiving $50\,mg\,kg^{-1}$ this type of lesion was seen in one animal only. Comparison of groups with focal darker areas in the colonic mucosa showed significant differences between positive control and D-002-treated groups. The wet weight of the caecum tissue from D-002-treated guinea-pigs was significantly lower than that of controls (Table 1).

Microscopic changes

In all animals of the positive control group the macroscopic focal dark lesions contained areas of mucosa heavily infiltrated by inflammatory cells, mainly macrophages but also including polymorphonuclear leukocytes. Breach of the surface epithelium was very frequently observed. In different sections of the colonic mucosa from the caecum to the rectum the histological changes included small focal mucosal inflammatory cell infiltrates, crypt dilatation of glands, and crypt abscesses.

Sulphate polysaccharide in the mucosa

Toluidine-blue-stained sections from all control animals contained focal deposits of metachromatic staining material within the lamina propria of the large bowel. The deposits were larger and more frequent in the caecum and ascending colon. Material stained with metachromatic purple was also observed when surface epithelial cells were studied by light-microscopy under higher magnification.

In D-002-treated animals the frequency of the inflammatory infiltrate was significantly reduced. Only two animals receiving doses of $25\,mg\,kg^{-1}$ showed areas of caecum mucosa infiltrated by numerous inflammatory cells (macrophages and PMN) and two animals treated with $50\,mg\,kg^{-1}$ showed only relatively small amounts of this type of cell in some areas (Table 2). Toluidine-blue-stained sections from D-002-treated animals showed notably reduced amounts of carrageenan in the lamina propria and within surface epithelial cells.

When sections of caecum were measured to assess the extent of wall thickening as an index of oedema or injury, a significant reduction was observed in the D-002-treated groups compared with control animals (Table 1). The histopathological index showed that damage in animals treated with D-002 was significantly less than in the control group. The overall score for the control group was 376 whereas for the D-002-treated groups they were 275 and 267 (Table 3).

Discussion

Low molecular weight, degraded, hydrolysed carrageenan has been shown to cause inflammation and ulceration of the caecum and colon of labora-

Table 3. Effect of administration of D-002 on the pre-ulcerative phase of carrageenan-induced colonic ulceration in guinea-pigs, as measured by the number of macroscopic lesions and by histologically assessed damage to the caecum mucosa.

Group	Dose ($mg\,kg^{-1}$)	Number of animals with macroscopic lesions	Histological index	
			Number	Mean ± s.d.
Control	–	8	376	2·35 ± 0·1
D-002	25	2*	275	1·71 ± 0·4‡
D-002	50	1*	267	1·63 ± 0·5‡

Results of the histological index are expressed as the overall total score and as the mean ± s.d. of results from eight animals. *$P < 0.005$ (Fisher's exact probability test), ‡ $P < 0.05$ (Mann-Whitney U-test), significantly different from control.

tory animals (Watt & Marcus 1971; Benitz et al 1973; Grasso et al 1973; Engster & Abraham 1976). In this work using an acute experimental model with a pre-ulcerative phase of three days (Marcus et al 1989) our results were similar to those aforementioned.

Administration of D-002 to guinea-pigs with carrageenan-induced colonic ulceration significantly reduced the wet weight of 5-cm caecum segments compared with controls. The wet weight of the inflamed colonic tissue is considered a reliable and sensitive indicator of the severity and extent of the inflammatory response (Rachmilewitz et al 1989). Administration of 25 or $50\,mg\,kg^{-1}$ D-002 for three days to guinea-pigs with carrageenan-induced colonic ulceration significantly reduced this type of lesion.

The histopathological index of the positive control group was significantly greater than for guinea-pigs treated with D-002. Similarly, a significant reduction of macrophages and PMN was observed in these D-002-treated animals and the colonic-wall thickness was also significantly reduced, indicating reduced oedema and tissue injury (Buell & Berin 1994).

It has been demonstrated that the colonic epithelium in the guinea-pig is capable of absorbing macromolecules (Marcus et al 1992). Hydrolysed carrageenan affects intercellular junctional integrity before damaging the cell membrane itself (Ling et al 1988). These authors also suggest that free radicals such as hydrogen peroxide could be involved in the intestinal inflammation induced by hydrolysed carrageenan. Treatment of the guinea-pigs with D-002 resulted in a reduction in the amount of sulphated polysaccharide within surface epithelial cells and in the lamina propria from the caecum to the rectum, suggesting a reduction of the altered permeability induced by carrageenan, or reduced absorption, or both.

PMN might be involved in the development of colonic injury, as has been demonstrated in many models of gastric (Kvietyz et al 1990; Wallace et al 1990) and small intestinal injury (Grisham et al 1990; Kubes et al 1991)—the occurrence of increased numbers of mucosal PMN in many forms of naturally occurring and experimental colitis has frequently been observed (MacPherson & Pfeiffer 1978; Morris et al 1989). The basis of the effect of D-002 demonstrated in this model could be either its capacity to prevent the migration of macrophages and PMN to the site of the injury, and the related release of toxic mediators such as leukotrienes by these inflammatory cells, or the inhibition of free radical formation induced by PMN and macrophages involved in the carrageenan-induced damage as a consequence of the integral action on the intercellular junctional integrity and membrane in the carrageenan-induced injury.

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