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Effect of rebamipide on the colonic barrier in interleukin 10 deficient mice

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ABSTRACT

Our aim was to study the effect of a mucosal protective agent, rebamipide, on the colonic barrier and the immune response in colitis-prone interleukin-10 deficient (IL-10^{-/-}) C57BL/6 mice infected with *Helicobacter hepaticus*. After sacrifice, in all mice, control, or previously infected with *H. hepaticus*, or previously infected and treated with rebamipide enema, a histological examination of colonic samples was performed, intestinal permeability was studied in Ussing chamber, and mesenteric lymph node proliferation and cytokine secretion were measured. Mice treated with rebamipide, presented a reinforcement of the distal colonic epithelial barrier, an increase of mesenteric lymph node cells proliferation and of IFN γ and IL-12 secretion. These results indicate that in IL-10^{-/-} mice with mild colitis, rectally administered rebamipide reinforces the distal colonic barrier and has a slight Th1 immunostimulatory effect on mesenteric lymph node cells. These properties could be helpful in the management of some inflammatory bowel diseases.

Keywords: rebamipide, intestinal permeability, colitis, interleukin 10, immune response

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), have multifactorial etiologies involving genetic, environmental, and immunological factors. One of the constant features in IBD is the increase in intestinal permeability (1, 2). Under normal conditions, the intestinal epithelium forms a barrier that simultaneously regulates the absorption of nutrients and water, and prevents the uptake of bacteria and bacterial products from the intestinal lumen. An impaired intestinal barrier function is observed in patients with CD (3, 4) but also in healthy relatives of these patients (5). The unrestricted passage of bacterial antigens from the intestinal lumen may induce the chronic activation of mucosal immune cells, a critical component of the inflammatory process associated with IBD.

Current treatment of IBD have focused on drugs able to decrease inflammation but the question of limiting antigen access to the submucosa by reinforcing the epithelial barrier has not been addressed. Rebamipide is a mucosal protective agent which, in addition to stimulating the production of endogenous prostaglandins in the gastric mucosa and improving ulcer healing (6), exerts a positive effect on the gastric barrier function. It reinforces the barrier's integrity in basal and inflammatory conditions and it inhibits the increase in macromolecular transepithelial transport induced by *Helicobacter pylori* (7, 8). Experimental data have shown that rebamipide can prevent dextran sulfate sodium-induced colitis in rats (9). In addition, in a human case report, rebamipide exhibited a beneficial effect on a proctitis type of ulcerative colitis (10).

Murine models of enterocolitis mimicking IBD, have been described (11, 12). Interleukin-10 deficient (IL-10 $-/-$) mice infected with *H. hepaticus* have been shown to develop a moderate

to severe inflammation of the large bowel (13). We hypothesised that in this model of colitis the reinforcement of barrier function and modulation of the intestinal mucosal immune system responses to luminal antigens would be a good therapeutic approach. Therefore, our aim was to study the effect of rebamipide on intestinal permeability and immune response in IL-10^{-/-} mice infected with *H. hepaticus*.

METHODS

Experimental protocol

Forty female IL-10-deficient C57BL/6 mice (B6.129P2-IL-10^{tm1Cgm}/Cr1) obtained from Charles River Laboratories, L'Arbresle, France (aged 5 weeks at the beginning of the experimental protocol) were used in this study. They were housed 12 per cage, at 20°C under specific pathogen free conditions during two weeks and then shifted to conventional litter and to a standard non-sterile diet.

Altogether, 4 groups of mice were studied: *Group I = Control group (n = 12)*: IL-10-deficient non-infected mice, *Group II = Infected group (n=12)*: IL-10-deficient mice infected with *H. hepaticus* (strain CCUG 33637, ATCC 51448) by three oral gavages with 2×10^9 CFU in 500 μ l at the age of 5 weeks, *Group III = Rebamipide group (n=12)*: IL-10-deficient mice infected with *H. hepaticus* and then, starting from one week after infection, treated daily with rebamipide enema (300 μ g of rebamipide in 100 μ l of carboxy-methyl cellulose used as a vehicle) during 9 weeks; *Group IV = Placebo group (n=4)*: IL-10-deficient infected mice, receiving rectally 100 μ l of the vehicle carboxy-methyl cellulose during 9 weeks instead of rebamipide enema.

H. hepaticus infection was confirmed by semi-nested PCR in stools. After DNA extraction from the fecal samples using the QIAamp® DNA stool mini kit (Qiagen) according to the manufacturer's instructions, a PCR was performed using primers F₂ 16S and R₄ 16S, detecting conserved bacterial 16S ribosomal DNA(14). These two oligonucleotides, produced an amplified product of 1490 pb. 2.5 μ l of the fecal DNA preparation was added to a 25 μ l (final volume) reaction mixture containing 1X reaction buffer (with 15 mM MgCl₂), 0.5 μ M each of the two primers, 200 μ M each deoxynucleotide, 0.1 μ g of bovine serum albumin per μ l and 0.025 U/ μ l of *Taq* polymerase (Promega, Charbonnières les Bains, France). The following conditions were used for amplification: an initial denaturation at 94°C for 5 min

was followed by 40 cycles (denaturation at 94°C for 30s, annealing at 55°C for 30s, elongation at 72°C for 1min20s) and a final elongation at 72°C for 7 min. After purification with Sephacryl™ S-400 high resolution (Amersham Biosciences, Saclay, France) column, a second set of primers was used for the amplification of a *Helicobacter spp* specific 16S ribosomal DNA fragment. These two oligonucleotides, 5'GCTATGACGGGTATCC3' (C₉₇) and 5'GATTTTACCCCTACACCA3' (C₉₈), produced an amplified product of 398 pb. The reaction mixture was the same as this used for the first amplification but without bovine serum albumin. After a denaturation at 94°C for 5 min, 40 cycles (denaturation at 94°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 30s) and a last elongation at 72°C for 7 min were performed. PCR was still positive in all infected mice 4 weeks after infection.

Throughout the protocol, mice were weighed twice a week and their general behaviour was recorded.

Experiments were conducted in accordance with the ethical guidelines of the French Veterinary Department.

Clinical and histological examination

At the end of the treatment protocol, the mice (aged 16 weeks) were sacrificed, and MLN and the entire colons were obtained. Proximal (caecum) and distal colonic samples were fixed in 4% formaldehyde for histologic examination while other fragments were opened along the mesenteric border and used to study permeability in Ussing chambers.

Microscopic score

Fixed tissues were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Sections were examined blindly by a single pathologist (A.R.). Results were expressed using a histological index ranging from 0 to 4 as previously described (15). This index is based on the degree of epithelial layer erosion, goblet cell depletion, and inflammatory cell

infiltrate [0=normal; 1=minimal evidence of inflammatory infiltrate; 2=significant evidence of inflammatory infiltrate (cryptitis, crypt abscesses); 3=significant evidence of inflammatory infiltrate with goblet cell depletion; 4=significant evidence of inflammatory infiltrate with erosion of the mucosa].

Epithelial barrier of the colonic mucosa

One caecal fragment and one distal colonic fragment from each mouse were laid on a millipore filter (8 μm pores) and mounted as flat sheets in Ussing chambers with an exposed surface area of 0.2 cm^2 . They were bathed on both sides with 1.5 mL of Ringer solution containing 5 mM mannitol, which was continuously thermostated, circulated, oxygenated and maintained at pH 7.4 with 5% CO_2 / 95% O_2 . The mucosal and serosal bathing solutions were connected via agar bridges to calomel electrodes for measurement of the transepithelial potential difference (PD) and to Ag-AgCl electrodes for current (ΔI) application. The tissues were kept under open-circuit conditions and electrical measurements were performed using a DVC 1000 voltage/current clamp (World Precision Instruments, Aston, U.K.). The tissues were regularly clamped at 1 mV to measure the ΔI and calculate the electrical resistance (R) according to Ohm's law ($\text{PD}=\text{R} \times \Delta I$).

Horseradish peroxidase (HRP, MW 40 kDa) was used as a soluble protein marker of the transcellular transport pathway (transcytosis). Mannitol was used as a small molecular tracer (MW 182 daltons) of the paracellular pathway. HRP (0.4 mg/ml), ^3H -HRP (37 kBq/ml) and ^{14}C -mannitol (12.2 kBq/ml) were simultaneously added to the mucosal compartment bathing intestinal fragments. Samples (800 μl) were taken from the serosal compartment at 10, 30, 50, 70, 90 and 110 min and replaced by fresh Ringer solution. Intact HRP fluxes from the mucosal to the serosal compartment (JHRP_i) were determined by enzymatic assay (16) on 200 μl of serosal samples. Total HRP fluxes (intact + degraded = ^3H -equivalent HRP fluxes) and

¹⁴C-mannitol fluxes were assessed on 500 µl serosal samples by counting the ³H and ¹⁴C radioactivity respectively, using liquid scintillation photometry (Kontron, betamatic) after double-labelling correction. Degraded HRP fluxes were calculated as ³H-equivalent HRP fluxes minus intact HRP fluxes. Mean steady-state fluxes obtained from 50 to 110 min are presented in figure 3.

Activation status of mesenteric lymph node cells

Mesenteric lymph nodes were collected at sacrifice and single cell suspensions were prepared in Cerottini culture medium (DMEM glutamax supplemented with 8% heat-inactivated fetal calf serum, asparagine 36 mg/L, arginine 116 mg/L, folic acid 10mg/L, HEPES 1g/L, β-mercaptoethanol 0.05 mM, penicillin 100 U/ml, streptomycin 100 µg/ml, fungizone 1 µg/ml). Generally, the total cell count was about 20 x 10⁶ cells/mouse which were adjusted to 2 x 10⁶ cells /ml in the culture medium.

Lymphocyte stimulation test: ³H-thymidine incorporation

Basal and stimulated cell proliferation was analyzed using ³H-thymidine incorporation. Cells were seeded in 96-well culture microplates at 4 x 10⁵ cells/well (180 µl) and were stimulated or not in duplicate with 20 µl of soluble extract of *E. coli* or *H. hepaticus*, at the final concentration of 1 and 3 µg/ml, respectively.

Soluble *E. coli* extract was prepared from cultures of *E. coli* isolated from human flora. The bacteria were harvested and washed thoroughly in PBS, and their lysis was performed by sonication at 4°C. Cell debris were removed by centrifugation at 8,000 x g for 30 min at 4°C, and the supernatant was sterile filtered. Its protein content, measured by the Bradford assay (Pierce, France), was 1.2 mg/ml. LPS concentration of this extract was measured using the Limulus ameocyte lysate (Charles River Endosafe, Charleston, VA, USA). The soluble extract contained 8.6 µg/ml of LPS, leading to the final concentration of 86 ng/ml in culture

wells. Soluble *H. hepaticus* extract was prepared from *H. hepaticus* strain cultures according to the same protocol. There was no detectable LPS in this extract (< 80 pg/ml). After a 72 hour incubation in the presence of soluble *E. coli* or *H. hepaticus*, [methyl-³H] thymidine (15 kBq/well) was added. ³H-labelled DNA was extracted 18 hours later using a cell harvester (Inotech, Perkin-Elmer). Filters were counted using a 1450 Microbeta Wallac Trilux microplate reader (Perkin-Elmer Life Sciences, Paris, France). Results are expressed both as counts per minute (cpm)/well and as stimulation index (SI), i.e. cpm ratio (stimulated cells / non-stimulated cells).

Cytokine secretion: ELISA and flow cytometry

ELISA: MLN cells were also seeded on 24-well culture plates (approximately 2×10^6 cells/well) in 1 ml of Cerottini culture medium with and without stimulation by soluble *E. coli* (1 µg/ml), and *H. hepaticus* (1 µg/ml) in duplicate. After 72 hours, supernatants were collected and frozen at -20°C until assayed. Four cytokines were assayed: IFN γ , TNF α , IL4 and IL12 using duoset ELISA kits (R&D, Abingdon, UK).

Flow cytometry: IFN γ secretion by MLN cells in basal conditions without stimulation was assessed by flow cytometry using a mouse IFN γ secretion assay (Miltenyi Biotec). MLN samples were seeded on 24-well culture plates (10^6 cells/well) in 1 ml of RPMI containing 5% mouse serum for 16 hours. MLN cells were washed in cold buffer, capture antibody against IFN γ /CD45 was added for 5 min on ice, then the samples were incubated for 45 min at 37°C under slow continuous rotation to allow cytokine secretion. Samples were washed and treated with anti-IFN γ APC-conjugated detection antibodies for 10 min on ice. The cells were counterstained with monoclonal antibody against CD3-PE, CD4-FITC or CD3-PE, CD8-FITC.

Activation markers on T cells

CD44 and CD62L markers were used to measure T cell activation. In activated cells, CD44 expression is increased and CD62L expression is decreased. MLN cells were stained with anti-CD3-PE and anti-CD44-FITC antibodies (Becton Dickinson, Le pont de Claix, France). CD62L staining was done using a biotinylated anti-CD62L antibody which was detected by a PE-cyanin7- Streptavidin conjugate (Becton Dickinson).

Statistical analysis

Statistical analysis was performed using the SAS package. The results are expressed as mean \pm SD and comparison of different parameters among the groups was performed by using analysis of variance. The general linear model procedure was also used for multiple group-to-group comparisons. Because of a small number of mice in placebo group, comparisons with this group were performed using non-parametric tests (Wilcoxon and Kruskal-Wallis). The differences were considered significant for $p < 0.05$.

RESULTS

Body weight curves

Only slight and non-significant differences in the weight gain during the experimental protocol were noted among the groups (Figure 1). Rebamipide treatment did not modify the growth rate as compared to Control- and Infected non-treated- mice.

Microscopic score

The microscopic alterations (scores not exceeding 2) observed in the caecum and distal colon were mild in all mice groups, as summarized in Figure 2. In infected mice, the microscopic score in the distal colon was higher than in control mice (2.10 ± 1.29 vs. 0.82 ± 0.60 , $p < 0.01$). No significant differences in microscopic score were observed between the mice treated with rectal rebamipide and the infected non-treated mice or placebo- treated mice.

Epithelial barrier function

Paracellular permeability

A reinforcement of the distal colonic barrier in mice treated with rebamipide enema was observed. This was attested by an increase in electrical resistance ($R = 63 \pm 25 \Omega/\text{cm}^2$) and a decrease in mannitol fluxes ($J_{\text{Man}} = 57 \pm 25 \mu\text{mol}/\text{h}\cdot\text{cm}^2$) as compared to Control mice ($R = 40 \pm 11 \Omega/\text{cm}^2$, $p < 0.05$ and $J_{\text{Man}} = 117 \pm 32 \mu\text{mol}/\text{h}\cdot\text{cm}^2$, $p < 0.0005$) and Infected mice ($R = 41 \pm 11 \Omega/\text{cm}^2$, $p < 0.05$ and $J_{\text{Man}} = 109 \pm 43 \mu\text{mol}/\text{h}\cdot\text{cm}^2$, $p < 0.003$) (Figure 3A). In contrast, no significant differences in the parameters reflecting epithelial integrity were found in the caecum ($R = 45 \pm 8$, 43 ± 11 , 42 ± 15 and $51 \pm 15 \Omega/\text{cm}^2$, $J_{\text{Man}} = 68 \pm 22$, 55 ± 23 , 53 ± 21 and $61 \pm 18 \mu\text{mol}/\text{h}\cdot\text{cm}^2$, for Control-, Infected-, Rebamipide- and Placebo- groups, respectively).

Macromolecular transport

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A decrease in total ($J^3\text{H}$ -equivalent HRP)- and degraded (JD) - HRP fluxes across the distal colonic mucosa was found in mice treated with rebamipide ($J^3\text{H}$ - equivalent HRP=1012 \pm 748 ng/h.cm², JD=977 \pm 758 ng/h.cm²) as compared to Control mice ($p<0.01$), Infected mice ($p<0.01$), and placebo mice ($p<0.05$). Intact HRP fluxes (JHRPi) were not significantly different between the groups (JHRPi = 29.3 \pm 24.7, 34.8 \pm 30.0, 35 \pm 36 and 17.2 \pm 9.0 ng/h.cm², for Control, Infected, Rebamipide and Placebo groups, respectively) (Figure 3B). No significant differences were observed in HRP fluxes in the caecum among the groups (data not shown).

Activation status of mesenteric lymph node cells

Lymphocyte stimulation test as measured by: ^3H -thymidine incorporation

Under basal conditions, MLN cell proliferation increased in the Rebamipide group (2180 \pm 2661 ^3H -cpm) as compared to the Infected group (830 \pm 830 ^3H -cpm, $p<0.04$), while no significant difference was found among the other groups (Figure 4A). After stimulation with soluble *H. hepaticus* antigens, there was again a significant increase in MLN cell proliferation in mice treated with rebamipide (4027 \pm 5817 ^3H -cpm) as compared to the Control (1503 \pm 1328 ^3H -cpm, $p<0.05$)- and Infected (1193 \pm 1223 ^3H -cpm, $p<0.03$)- mice (Figure 4B). This difference, however, was not found when results were expressed as stimulation indexes (1.09 \pm 0.23, 1.33 \pm 0.25, 1.33 \pm 0.61 and 1.14 \pm 0.16 in Control, Infected, Rebamipide and Placebo groups respectively, $p>0.05$), indicating that the basal and stimulated responses to *H. hepaticus* increased proportionally in the Rebamipide group. No significant difference was observed in MLN proliferation after stimulation with soluble *E. coli* antigens.

Cytokine secretion as measured by ELISA

Under basal conditions (Figure 5A), there was a clear increase in IFN γ secretion by MLN cells in the Rebamipide group (36.4 \pm 93 pg/ml) as compared to Control (4.1 \pm 13 pg/ml)- and

Infected (2.9 ± 11 pg/ml)- groups ($p < 0.02$). Similarly, rebamipide-treated mice presented a higher secretion of IL-12 (105 ± 90 pg/ml) than Infected mice (38 ± 38 pg/ml, $p < 0.01$). The basal IL-4 secretion achieved a higher level in Control mice (74 ± 77 pg/ml) than in all of the other groups (2.8 ± 7.8 , 4.8 ± 4.0 and 4.8 ± 4.8 pg/ml for Infected, Rebamipide and Placebo groups, respectively, $p < 0.004$). After stimulation with soluble *H. hepaticus* antigens (Figure 5B), a significant increase in IFN γ (148 ± 238 pg/ml) and IL-12 (239 ± 220 pg/ml) secretion was observed in the Rebamipide group, as compared to all of the other groups, ($p < 0.05$). The IL-4 secretion remained the highest in Control mice (54.3 ± 47 pg/ml) in comparison to Infected mice (19.2 ± 25 , $p < 0.004$) or Placebo-treated mice (0 ± 0 , $p < 0.01$). After stimulation with soluble *E. coli* antigens, a similar increase in IFN γ secretion in the Rebamipide group was observed (3474 ± 3289 pg/ml versus 103 ± 169 , 232 ± 383 and 57 ± 90 pg/ml, for Control, Infected, and Placebo groups, respectively, $p < 0.0001$).

IFN- γ secretion as measured by flow cytometry

The percentage of IFN γ -secreting MLN cells in basal conditions, measured by flow cytometry, is presented in Figure 6. A significant increase in both subsets, CD4 $^{+}$ and CD8 $^{+}$, of T cells secreting IFN γ was observed in mice treated with rebamipide enemas (IFN γ CD4 $^{+}$: $6.1 \pm 2.0\%$ and IFN γ CD8 $^{+}$: $4.1 \pm 2.0\%$) as compared to the Control group (IFN γ CD4 $^{+}$: $1.2 \pm 2.0\%$ and IFN γ CD8 $^{+}$: $0.76 \pm 0.80\%$, $p < 0.0001$) and the Infected group (IFN γ CD4 $^{+}$: $1.1 \pm 0.9\%$ and IFN γ CD8 $^{+}$: $0.46 \pm 0.20\%$, $p < 0.0001$).

Expression of T cell activation markers CD44 and CD62L as measured by flow cytometry

No significant difference was found in the expression of CD44 and CD62L evaluated by flow cytometry (mean fluorescence intensity) among the four groups (CD44: 27.9 ± 8.5 , 27.5 ± 10.2 and 22.9 ± 9.3 in the Control, Infected and Rebamipide groups, respectively; CD62L:

20.7 ± 3.7, 27.4 ± 9.1 and 23.0 ± 4 in the Control, Infected and Rebamipide groups, respectively).

DISCUSSION

This study confirms the protective effect of rebamipide on the intestinal barrier, namely its capacity to reinforce the epithelial barrier capacity and to decrease the macromolecular transport across this barrier. At the same time, it demonstrates for the first time the immunostimulatory properties of rebamipide, capable of stimulating MLN cell proliferation and cytokine secretion.

The beneficial effect of rebamipide with respect to the epithelial barrier, already recognized for gastric and small intestinal mucosa (7, 17), has now been shown for colonic mucosa. This effect concerns both paracellular and transcellular permeability. The fact that the improvement of barrier function observed after treatment with rebamipide enema was observed in the distal colon only, and not in the caecum, strongly points to the topical mechanism of action of rebamipide. The mechanism of its action on epithelial permeability, mostly studied in the stomach, is not completely understood. It could be related to rebamipide's capacity to act as a scavenger of cytokine-induced hydroxyl radicals (18) or to induce prostaglandin production (19). A direct effect of the drug on the epithelial cells and/or inter-epithelial tight junctions, could also play a role.

The protective effect of rebamipide on the colonic epithelial barrier and transepithelial protein absorption could account for its beneficial effect on a proctitis type of ulcerative colitis in man (10) or dextran sulfate sodium-induced colitis in rats (9). Indeed, in IBD, an increased epithelial permeability to luminal antigens can precede inflammatory lesions (20) and trigger mucosal inflammation. An increase in endosomal uptake of proteins, like HRP, in the histologically non-involved ileum of patients with CD, seems to be related to TNF- α expression (21). Moreover, anti-TNF- α antibodies, infliximab, can restore the epithelial barrier alteration in CD (22).

On the other hand, this study demonstrates the capacity of rebamipide to stimulate an immune response of MLN cells. This is attested by its capacity to induce MLN cell proliferation and also pro-inflammatory cytokines (IFN γ and IL-12) secretion by these cells, both under basal conditions and after stimulation with *H. hepaticus*. Taking into account the reinforcement of intestinal barrier by rebamipide, apparently associated with a decreased intestinal antigen, the higher proliferation of MLN cells in response to *H. hepaticus* antigens in rebamipide-treated mice is not easy to explain. It is possible, however, that specific immune response towards the infectious bacteria is increased not due to the higher amount of infectious antigens crossing the epithelial barrier but rather to the lower threshold of the immune response by MLN cells induced by rebamipide. The rebamipide-enhanced cytokine secretion was demonstrated by ELISA and confirmed by flow cytometry. The lack of effect of rebamipide on the T cell stimulation markers, CD44 and CD62L, suggests that these markers are less sensitive parameters of T-cell stimulation than cytokine secretion, at least in our model. The immunostimulatory properties of rebamipide observed in this study are in contrast to the previous studies where an inhibitory effect was observed on immune response, both *in vitro* (23-27), and *in vivo* (9, 28-30) . This apparent contradiction may be explained by differences in the models used. In most of the studies cited above, the effect of rebamipide on immune response was tested under strong inflammatory conditions. In our experimental setting, the mice presented only a very slight colitis with histological scores reaching 2.0 at maximum, and low basal IFN γ and IL-12 secretion which situates our model under sub-physiological conditions rather than pathological conditions. It is, therefore, possible that the effect of rebamipide observed in this study reflects an immunomodulatory effect similar to what is observed with probiotics, i.e. down regulation under inflammatory conditions and up regulation under physiological conditions (31-36). It is also possible that the known down regulatory effect of rebamipide on immune response does not appear in this model of IL-10-deficient mice

because the drug may need IL-10 to reveal this effect. Indeed, rebamipide has been shown to strongly inhibit IL-10 secretion by human peripheral blood mononuclear cells *in vitro* (23). On the other hand, it is important to point out that the maximal increase in IFN γ secretion induced by rebamipide in this study (4 ng/ml), was lower than the concentration necessary to significantly damage the epithelial barrier (10 ng/ml), as assessed *in vitro* (37). In fact, our results showed that rebamipide-treated mice presented a reinforcement of the intestinal barrier as compared to the other groups. This indicates that the relative increase in IFN γ secretion induced by rebamipide had no deleterious effect on the barrier.

A slight stimulation of Th-1 immune response by rebamipide in this model of Th1-mediated colitis in IL-10 knock-out mice, should logically be associated with exacerbation of colitis. However, no difference was observed in this study in the degree of colitis between rebamipide-treated and non-treated mice. This may be related, on one hand, to the generally low degree of colitis observed in all mice, not allowing to see any significant differences in colitis score among the groups and, on the other hand, to the relatively low degree of stimulation of Th-1 response by rebamipide, as mentioned above.

It is conceivable that this slight stimulation of Th1-type immune response by rebamipide may counteract Th2 immune responses. This could explain the beneficial effect of rebamipide described in proctitis type of ulcerative colitis in man (10), a disease associated with Th2-stimulation (38, 39). Clinical studies are necessary to confirm this hypothesis.

We did not observe any effect of rebamipide on colonic histology. This may again be related to the low degree of inflammation obtained in all animals, not allowing to evidence any difference between treated and non-treated mice. Indeed, the model of IBD in IL-10-deficient mice that we chose, is highly susceptible to environmental factors (housing, food) and genetic factors (mice strains) (40). Although Kullberg *et al.* published several studies where

inflammation was increased in the presence of *H. hepaticus* (13), other authors have failed to induce a marked colitis in these mice even after colonization with *H. hepaticus* (41).

In conclusion, this study shows that under sub-normal conditions of mild colitis, rectally administered rebamipide exerts a direct beneficial effect on the large bowel epithelial barrier integrity and function. It restrains the paracellular pathway and decreases protein absorption across the barrier. It also reveals an immuno-stimulatory effect on mesenteric lymph node cells, stimulating secretion of Th1-type cytokines, IFN γ and IL-12. This improvement of the intestinal barrier, together with the capacity of rebamipide to modulate immune responses, may be a new therapeutic approach potentially useful in the clinical management of IBD, especially those associated with Th2-stimulation like ulcerative colitis. Future clinical studies are necessary to test its utility in such a group of patients.

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LEGEND TO FIGURES

Figure 1

Body weight curve of IL-10 deficient control mice (Control), mice infected with *Helicobacter hepaticus* (Infected), mice infected and treated rectally with rebamipide (Rebamipide) or with placebo (Placebo). No significant difference was found among the groups.

Figure 2

Microscopic score of colitis in the caecum and distal colon in IL-10 deficient control mice (Control), mice infected with *Helicobacter hepaticus* (Infected), mice infected and treated rectally with rebamipide (Rebamipide) or with placebo (Placebo), according to McCarthy score (0 to 4). Infected mice presented a higher score than control mice. Treatment with rebamipide had no effect on histological score of colitis. # : $p < 0.001$ as compared to control mice.

Figure 3

Effect of rebamipide on the distal colonic epithelial barrier in IL-10 $-/-$ mice, measured *ex vivo* in Ussing chamber (mean \pm SD):

A: Electrical resistance (R) and mannitol fluxes (JMan) as markers of paracellular pathway,

B: Macromolecular (HRP) transport as marker of transcytosis

Decreases in paracellular permeability and HRP transcytosis were observed in rebamipide-treated mice, as compared to other groups

* $p < 0.05$ as compared to Control and Infected

$p < 0.01$ as compared to Control and Infected and $p < 0.05$ as compared to Placebo

Figure 4

Effect of rebamipide on proliferative response of MLN cells (mean±SD) in basal (A) and stimulated (B) conditions. A stimulatory effect of rebamipide on basal- and soluble *Helicobacter hepaticus*-stimulated - MLN cell proliferation was observed.

* p<0.05 as compared to Infected

Figure 5

Effect of rebamipide on inflammatory response of MLN cells: basal (A) and soluble *Helicobacter hepaticus*- stimulated (B) cytokine secretion assessed by ELISA (mean±SD). An increase in basal- and *H. hepaticus* stimulated- IFN γ and IL-12 secretion in rebamipide-treated mice was observed.

*: p<0.03 as compared to Control and Infected groups

#: p<0.01 as compared to Infected mice

§: p<0.004 as compared to all other groups

** : p<0.05 as compared to all other groups

§§: p<0.02 as compared to Infected and Placebo groups

Figure 6

Effect of rebamipide on inflammatory response of MLN cells: IFN γ secretion by T CD4 and T CD8 cells assessed by flow cytometry. An increase in % of IFN γ -secreting CD4+ and CD8+ cells in rebamipide-treated mice was observed.

*: p<0.0001 as compared to Control- and Infected- mice