Bacterial Overgrowth and Inflammation of Small Intestine After Carboxymethylcellulose Ingestion in Genetically Susceptible Mice

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Background: Detergents and emulsifiers added to food may destroy the mucus barrier, which normally isolates bacteria from the intestinal wall, and lead to chronic bowel inflammation in susceptible persons. We investigated the influence of 2% carboxymethylcellulose (CMC) on the biostructure of the intestinal microbiota in IL-10 gene-deficient mice.

Methods: Twenty to 27-week-old IL-10 gene-deficient mice received either 2% CMC solution ($n = 7$) or water ($n = 6$) orally for 3 weeks. Intestinal bacteria were investigated using fluorescence in situ hybridization in paraffin-fixed sections of the intestine.

Results: CMC-treated IL-10 gene-deficient mice demonstrated a massive bacterial overgrowth, distention of spaces between villi, with bacteria filling these spaces, adherence of bacteria to the mucosa, and migration of bacteria to the bottom of the crypts of Lieberkuehn. Leukocytes migrated into the intestinal lumen in 4 of the 7 CMC mice. The changes were similar to those observed in Crohn's disease in humans and were absent in control animals.

Conclusions: CMC induces bacterial overgrowth and small bowel inflammation in susceptible animals. Because of its ubiquity in products and its unrestricted use in food of the industrial world, CMC is an ideal suspect to account for the rise of IBD in the 20th century.

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Key Words: carboxymethylcellulose, biostructure, intestinal microbiota

The healthy mucosa is protected from contact with potentially harmful bacteria such as *Bacteroides, Enterobacteriaceae, Enterococci* or *Clostridium perfringens* by an impenetrable, highly viscous mucus barrier. Before bacteria can adhere to and invade the mucosa, they must cross the 40–240-μm thick mucus layer, which is normally free of bacteria.[1] As long as the mucus barrier is intact, the organism ignores the high concentrations of bacteria in the lumen of the large intestine and tolerates the bacteria without an immune response.[2] The mucus barrier is deficient in patients with inflammatory bowel disease (IBD), where bacteria contact the mucosa and build polymicrobial biofilms on the epithelial surface prior to the inflammatory response.[3] The reasons for this deficiency are unclear.

Since the beginning of the 20th century there has been a steady increase in the incidence of both Crohn's disease (CD) and ulcerative colitis (UC).[4] In parallel, detergents are increasingly utilized in households and emulsifiers are increasingly added to food. The "cleaning" effect of emulsifiers and detergents on the colonic mucus has never been investigated. The most prominent emulsifying substance added to the vast majority of food products is carboxymethylcellulose (CMC).

The present study investigates the changes in the biostructure of the intestinal microbiota after 3 weeks of daily exposure to CMC in IBD-susceptible IL-10 gene-deficient mice.

MATERIALS AND METHODS

IL-10 gene-deficient mice were housed in the animal facility at the University of Alberta, Canada, under specific pathogen-free (SPF) conditions and were used for the study at ages 20 to 27 weeks. Seven mice were gavaged once a day with 100 μL of 2% CMC (Sigma, Oakville, ON, Canada) solution for 3 weeks. The control group consisted of 6 littermates who were gavaged with 100 μL water in the same manner. The mice were euthanized and the intestines were removed after 3 weeks of treatment. Ten-mm long sections of jejunum, ileum, and proximal and distal colon were fixed in Carnoy (6/6/1) solution and shipped to the Laboratory for Polymicrobial Infections in

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TABLE 1. Bacterial Numbers and Distribution Within the Small Intestine of Each IL-10 Gene-deficient Mouse

Mouse	Segment	Concentration of Bacteria in the Lumen x10^8/mL	Concentration of Bacteria Between Villi x10^8/mL	Bacterial Number per Single Villi	Percent of Villi Contacting Bacteria	Percent of the Surface of the Villi Covered with Bacteria	Number of Leukocytes Within the Lumen and Between Villi
Water-treated							
KOW1	Jejunum	0.001	0.01	5	4	0	0
KOW2	Jejunum	0.0001	0	0	0	0	0
KOW3	Jejunum	No lumen	0	0	0	0	0
KOW4	Jejunum	No lumen	0	0	0	0	0
KOW5	Jejunum	No lumen	0	0	0	0	0
KOW6	Jejunum	0.0001	0	0	0	0	0
KOW1	Ileum	0.1	0	0	0	0	0
KOW2	Ileum	0.4	0	0	0	0	0
KOW3	Ileum	0.006	0	0	0	0	0
KOW4	Ileum	No lumen	0	0	0	0	0
KOW5	Ileum	0.001	0	0	0	0	0
KOW6	Ileum	0.002	0	0	0	0	0
CMC-treated							
KOCMC7	Jejunum	0.001	0	0	0	0	0
KOCMC8	Jejunum	No lumen	0	0	0	0	0
KOCMC9	Jejunum	0.1	0.001	0	0	0	20
KOCMC10	Jejunum	8	0.1	120	80	55	0
KOCMC11	Jejunum	6	1	1	3	7	100
KOCMC12	Jejunum	No lumen	0.01	0	0	0	0
KOCMC13	Jejunum	No lumen	0	0	0	0	0
KOCMC7	Ileum	4	2	30	80	50	0
KOCMC8	Ileum	12	6	15	80	42	0
KOCMC9	Ileum	2	0.8	12	8	3	0
KOCMC10	Ileum	20	10	40	100	60	600
KOCMC11	Ileum	No lumen	0	1	4	1	0
KOCMC12	Ileum	30	8	30	20	25	600
KOCMC13	Ileum	6	12	3	25	17	0
Exact *P*-value*		0.003	0.006	0.011	0.009	0.004	0.231

*The variables listed in Table 1 showed a nonparametric distribution on 2-sample Kolmogorov-Smirnov testing, and hence all comparisons between distributions of values for water- and CMC-treated mice were made under the nonparametric assumption through the Mann-Whitney *U*-test. On account of the sample size exact, rather than ordinary asymptotic *P*-values were calculated.

Berlin, Germany.[1] The samples were embedded in paraffin, cut to 4-μm thick sections, fixed on glass slides, and hybridized with a set of 3 probes: Eub338-FITC (which recognizes all bacteria), MIB661-Cy3 (which recognizes the *Bacteroides* group in the mouse), and EREC (which recognizes the *Eubacterium rectale* group). Subsequently the slides were counterstained with DAPI. Bacteria were visualized with a Nikon microscope and camera in real colors as previously described.[1–3]

The results of the hybridization of the IL-10 gene-deficient mouse intestines were compared with results reported previously describing changes in the intestine of patients with CD.[3] Treatment of mice was approved by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta.

RESULTS

Control Animals

The small intestine of mice receiving water only had low numbers of bacteria within the intestinal lumen and no leukocytes (Table 1). Hybridization with the universal

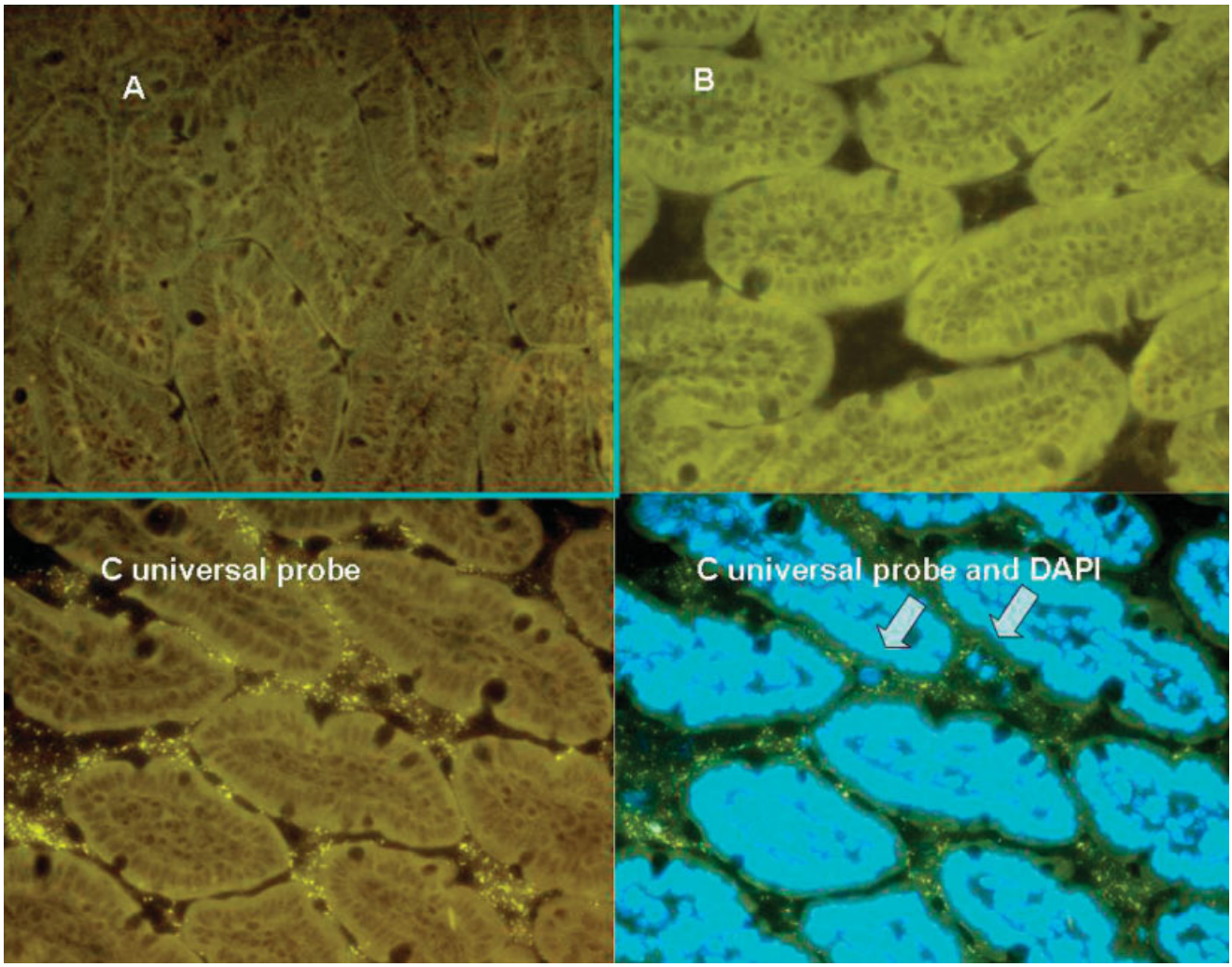

FIGURE 1. Jejunum of IL-10 gene-deficient mice (A: water-treated; B,C: CMC-treated mouse) hybridized with the universal Eub338-Cy3 probe (orange fluorescence) × 400. A: Water-treated mouse KOW3. No bacteria can be seen between villi, the walls of the villi touch each other, leaving no large spaces between them. B: CMC-treated mouse KOCMC7. No bacteria were detected between the villi; however, the spaces between the villi are generally distended. C, left: CMC-treated mouse KOCMC11. Bacteria are located between villi in high concentrations throughout the jejunum. C, right: Shows the same microscopic field as C, left with Eub338-Cy3 hybridization and DAPI counterstain overlaid. Leukocytes in small numbers (blue nuclei, white arrows) can be seen between villi and are surrounded by bacteria.

bacterial probe Eub338-Cy3 revealed concentrations between 0.001 to 0.0001 × 10^8/mL in the lumen of the jejunum and between 0.001 to 0.4 × 10^8 in the lumen of the ileum.

CMC-treated Mice

The concentrations of luminal bacteria and bacteria located between villi were significantly higher than in the control mice and ranged between 0.001 to 6 × 10^8/mL in the jejunum and 2 to 30 × 10^8/mL in the ileum ($P < 0.01$). Contrary to water-treated control mice, 4 of the 7 CMC-treated mice had leukocytes within the lumen of the intestine (Table 1; Figs. 1C, right; 2B); in 2 this was observed in the jejunum and in 2 other mice in the ileum. The distribution of leukocytes between jejunum and ileum was mutually exclusive: mice with a leukocyte response in the ileum had no leukocytes in the lumen of jejunum and vice versa. Leukocytes were absent in the lumen of both the jejunum and ileum in all water-treated mice. The most striking changes between the 2 groups were observed in spaces between villi and the crypts of Lieberkuehn. Only 1 water-treated mouse had detectable bacteria in these spaces (Table 1; Figs. 1A, 2A). This 1 mouse, KOW1, demonstrated maximal 5 bacteria surrounding 4 of 80

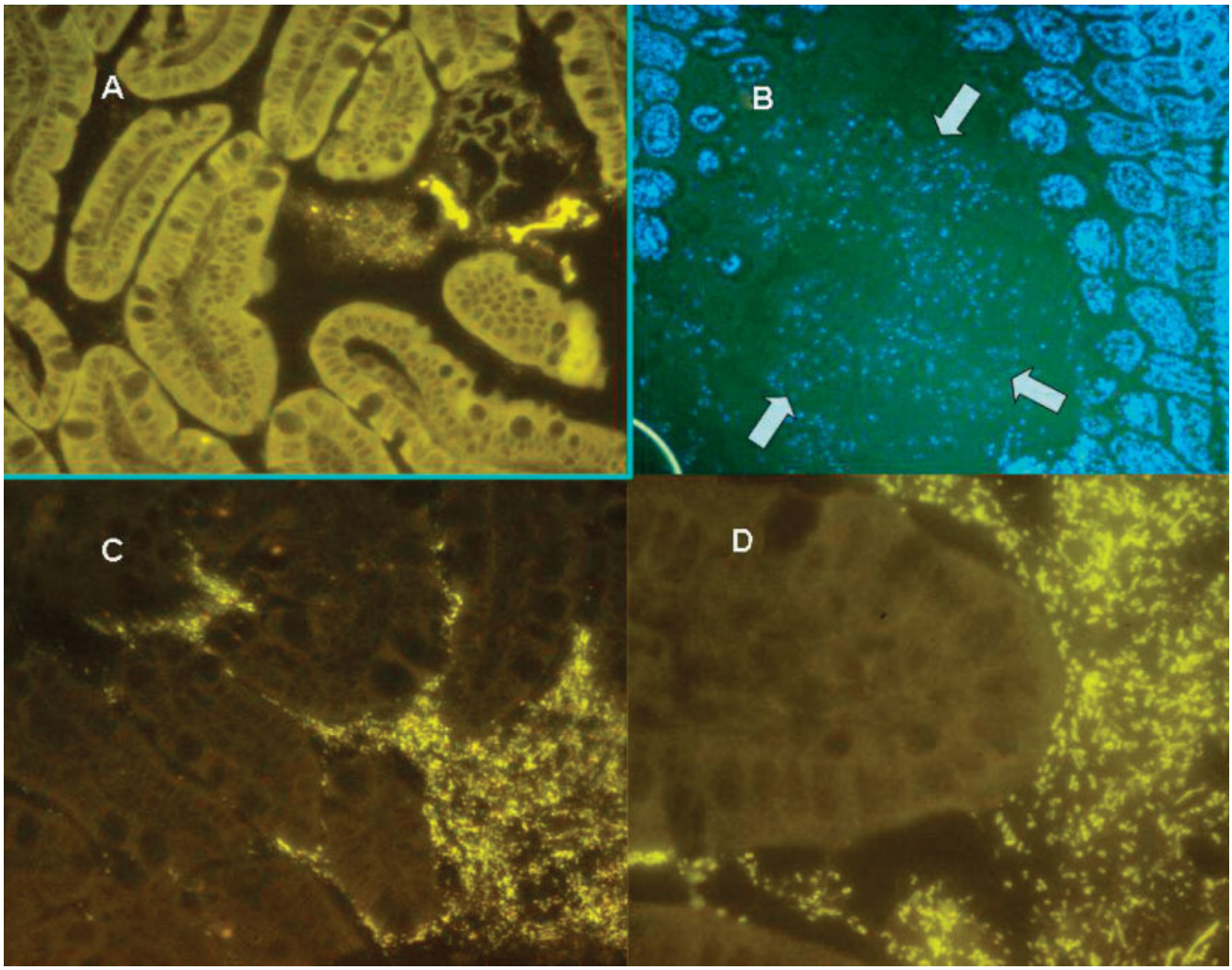

FIGURE 2. Ileum of IL10 gene-deficient mice (A: water-treated; B–D: CMC-treated mice) hybridized with the universal probe Eub338-Cy3 (A,C ×400, D ×1000) and DAPI counterstain (2B ×100). A: Mouse KOW2 treated with water only; single bacteria can be seen within the intestinal lumen, no bacteria were detected between villi or within the crypts of Lieberkuehn. B: Mouse KOCMC10; visible leukocytes have migrated into the intestinal lumen (large blue nuclei, white arrows). C: The same sample as in B at higher magnification shows the massive increase in the number of bacteria intraluminally with bacteria surrounding most of the villi, partially adhering to the surface of villi and migrating deep into the crypts of Lieberkuehn. D: Mouse KOCMC7; adherence of bacteria to ileal villi at higher magnification.

visible crypts. In contrast, all mice treated with CMC had broad gaps between villi (Figs. 1B, 2B,C). These gaps were filled with bacteria in 2 of the 7 CMC mice in the jejunum and in all 7 CMC-treated mice in the ileum. The mean bacterial number per villi in the ileum was 45, whereas on average 28% of the villi were surrounded by bacteria (Figs. 1C, 2C,D) in the CMC group. Bacteria between villi penetrated deeply into the crypts of Lieberkuehn and adhered to the epithelial surface in a patchy manner (Fig. 2D).

The morphological appearance of the highly concentrated bacteria adherent to villi in the ileum of the CMC mice together with concomitant migration of leukocytes into the adjacent mucosa regions resembled findings observed in patients with CD (Fig. 3B–D).

Moderate to severe distal colonic inflammation with concomitant migration of leukocytes into the intestinal lumen was observed in all mice, both the water- and CMC-treated mice. Because of the high individual variation in the degree of inflammation, we did not detect significant differences between these 2 groups (data not shown).

The microbiota detected in the small intestine of the CMC-treated animals were similarly composed as those of

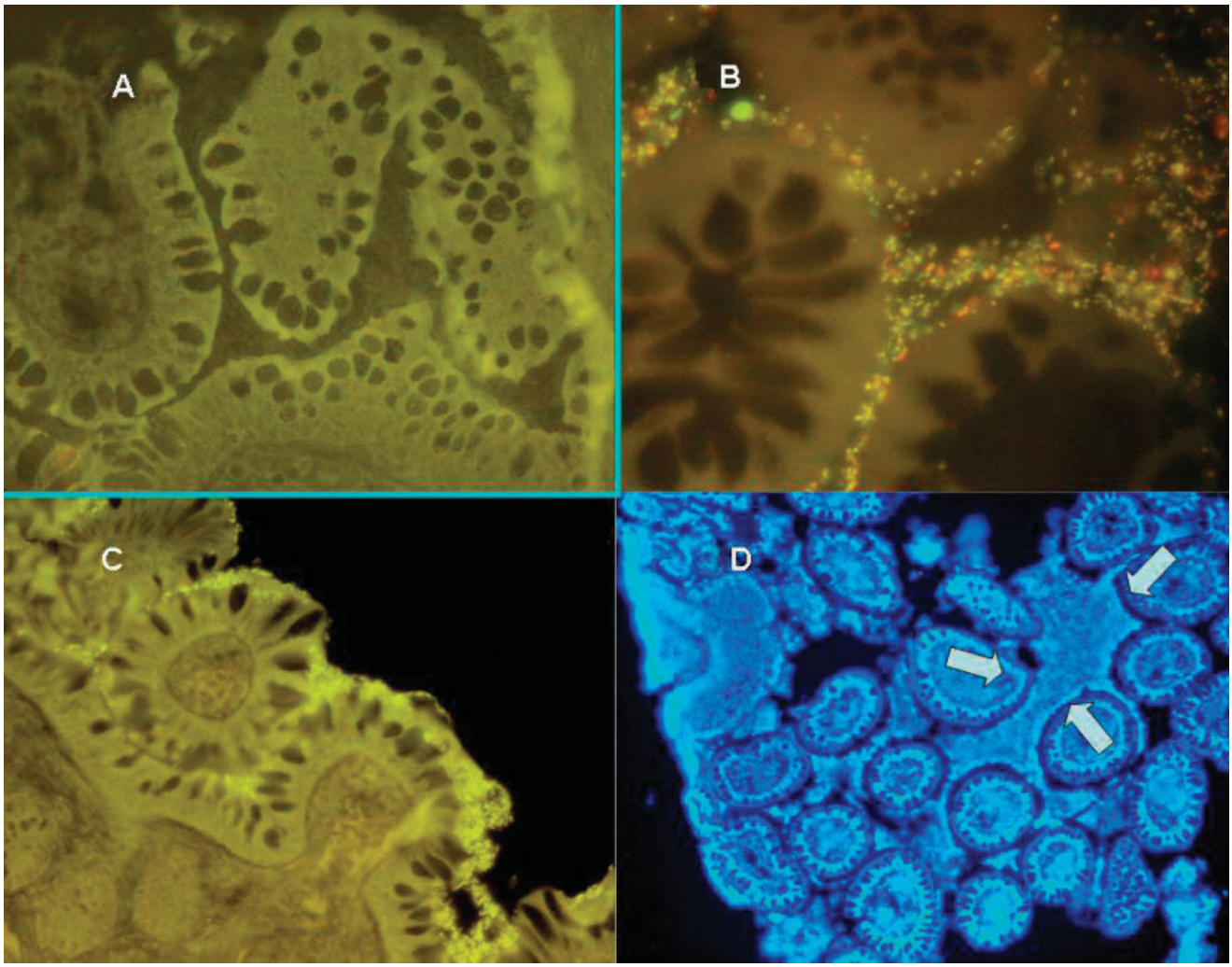

FIGURE 3. Ileal biopsies from a healthy person (A) and a patient with CD (B–D). A: No bacteria can be detected by hybridization with universal probe Eub338-Cy3 × 400 in a healthy control. The mucosa is covered with mucus. B: Multicolor FISH demonstrates adherence of different bacterial groups (Bac303-Cy3, orange fluorescence; *Bacteroides*, Eub338-FITC, green fluorescence; all bacteria, EREC-Cy5, red fluorescence; *Eubacterium rectale* group) ×1000. C: Hybridization with the universal probe demonstrates prolific adherence of bacteria to ileal villi in CD ×400. 3D: DAPI counterstain demonstrates a massive leukocyte migration into the spaces between villi in a patient with CD. The figures were selected from the database of the Laboratory for Polymicrobial Infections and Bacterial Biofilms. The corresponding analysis of the numeric data was previously published.[3]

the colonic microbiota. There was a higher portion of *Bacteroides* in the ileum and an overall lower amount of the *Eubacterium rectale* group in the jejunum and ileum.

DISCUSSION

CMC is extensively used in the food industry for its emulsifying and thickening properties—it is abundant and cheap. CMC is added to food to stabilize emulsions, for instance, in ice cream, to dissolve ingredients such as cacao in order to make perfect chocolate and sugar icing, to boost the flavor of the natural aroma and to keep bread fresh and soft. It can be found in toothpaste, chewing gum, a variety of baked goods, candies, sausages, ketchup, and other foods. It is a filling and stabilizing component of most pills and a main substitute for gluten in manufactured gluten-free products. CMC is everywhere in quantities that are larger than those administered to the mice in our experiment. The annual amount of CMC utilized by the food industry is steadily increasing. Because CMC, as all natural fibers, cannot be absorbed and is chemically inert, and since it has been broadly used worldwide for nearly 100 years without apparent negative effects, its health impact as a food additive is thought to be purely related to the water and viscosity environment within the intestine.

Presently, there are no quantitative restrictions on its use, and its addition to food does not even require that it be declared. This attitude is problematic. Reports indicate that emulsifiers can dramatically reduce the lethal dose of toxic substances when applied orally or to the skin in animal experiments reaching back to the 1930s of the 20th century.[5] However, all these indices were indirect and involved substances other than CMC. Our investigation is the first to demonstrate that CMC, on its own, can induce "CD-like effects" in animals with an aberrant genetic background. The harmful effects were observed in adult IL-10 gene-deficient mice, which are used worldwide as a model for the inflammation of IBD. Because of the lack of a major regulatory cytokine, the IL-10 gene-deficient mouse spontaneously develops a severe enterocolitis that resembles the colitis of IBD in humans.[6] The colitis is clearly mediated by intestinal bacteria, because colitis does not develop in germ-free mice of the same genetic background.[7] In contrast to the large intestine, which harbors extremely high concentrations of bacteria, the small intestine of the IL-10 gene-deficient mouse contains no relevant bacterial concentrations.[2] This might explain the lack of inflammation in the small intestine of these mice despite the general nature of the gene defect.

These findings were also reproduced in our water-treated mice. The mucus surrounding villi in the small intestines of 5 out of 6 water-treated mice were bacteria-free and showed no signs of inflammation, while colon and cecum had the characteristics of moderate to severe inflammation that are typically found in these mice. Although the changes in the large intestines of the CMC-treated mice were similar to those of controls, the situation in the small intestine of CMC-treated mice was markedly different. Bacterial concentrations in the ileum of 6 of 7 mice, who had received 2% CMC solution, were higher than 10^8/mL. This is comparable to bacterial concentrations otherwise exclusively observed in the colon. The lumen of the CMC-treated mice was distended; the spaces between villi and the crypts of Lieberkuehn were increased. Bacteria adhered to the surface of the villi and were often found at the bottom of the crypts of Lieberkuehn. In 4 of the CMC-treated mice large amounts of leukocytes had migrated into the lumen of the small intestine and in 2 mice bacterial infiltration of the epithelium was observed. In addition, 2 of the CMC mice also had high concentrations of bacteria in the jejunum. The changes observed in CMC mice were identical to those observed in ileal biopsies of patients with CD. We are aware that this similarity is just visual and descriptive; however, we are alarmed by the extent of the differences between CMC-treated and water-treated control mice. We feel that it is very important to make the medical community aware of the "harmless fiber" and stimulate further clinical investigation of this phenomenon. Because of its ubiquity in products and its unconsidered and uncontrolled use in the "industrial world" diet, CMC is an ideal suspect to account for the rise of IBD in the 20th century. The question of CMC's impact on health and disease needs to be quickly answered.

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