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RESEARCH ARTICLES

Carrageenan-induced innate immune response is modified by enzymes that hydrolyze distinct galactosidic bonds

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Abstract

The common food additive carrageenan (CGN) predictably induces intestinal inflammation in animal models. Mechanisms of CGN-induced nuclear factor κB and interleukin-8 (IL-8) stimulation include an immune-mediated pathway involving toll-like receptor 4 (TLR4) and B-cell lymphoma/leukemia 10 (BCL10) and a reactive oxygen species (ROS)-mediated pathway. To determine how the structure of CGN contributes to its initiation of inflammation through these two distinct mechanisms, we treated CGNs with galactosidases and carrageenases (CGNases) and determined the impact on IL-8 secretion and BCL10 production. Hydrolysis of CGN by the enzyme α-1→(3,6)-galactosidase significantly reduced increases in IL-8 and BCL10, but other galactosidases tested, including α-1→6-galactosidase, β-1→4-galactosidase and β-1→3,6-galactosidase, had no effect. In contrast, specific κ-CGNases or ι-CGNases, which hydrolyze β-1,4-galactosidic bonds, produced increases in IL-8 and BCL10 attributable to increased exposure of the immunogenic α-1→3-galactosidic epitope of CGN to TLR4. These results were consistent with induction of innate immune response by an interaction of TLR4 with the unusual α-D-Gal-(1→3)-D-Gal epitope present in CGN. Activation of the ROS-mediated pathway was unaffected by treatment of κ-CGN with either κ-CGNase (3 mg/L), α-1→(3,6)-galactosidase (20 mU/ml) or these enzymes in combination, indicating that changes in IL-8 production were attributable to the effects of induction of inflammation on the TLR4–BCL10-mediated innate immune pathway. These findings provide new information about the specificity of carbohydrate–protein interaction between CGN and TLR4 and may help to devise treatments that modify the immune reactivity induced by carbohydrate antigens.
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Keywords: Carrageenan; Inflammation; Food additive; Galactosidase; Carrageenase

1. Introduction

The sulfated polysaccharide carrageenan (CGN) has a long history of use as a food additive in the Western diet [1,2]. CGN, obtained from several species of red algae, is frequently incorporated into processed foods to improve texture and solubility. CGN consumption has increased steadily in recent decades [3,4]. In finished food products, it exists predominantly as an undegraded sulfated polysaccharide with a molecular weight of over 100,000, although contamination by lower-molecular-weight forms is common [5]. Processes such as heating, acid treatment, mechanical effects and bacterial digestion can all lead to the production of degraded lower-molecular-weight forms of CGN [1,2,6,7].

The major forms of CGN have in common their basic structure (consisting of an ideal disaccharide unit) composed of D-galactose residues alternatively linked by β-1→4 and α-1→3 glycosidic linkages (G and D units, respectively). CGNs are classified according to the number and position of sulfated ester (S) and by the occurrence of 3,6-anhydro bridges in α-linked residues (DA unit). Three major varieties are incorporated into food products in various combinations: κ-CGNs (DA-G4S), ι-CGNs (DA2S-G4S) and λ-CGNs (D2S6S-G2S) (Fig. 1) [8,9]. κ-CGNs and ι-CGNs form thermoreversible gels in aqueous solutions, the rigidity of which decreases strongly with the degree of sulfation. In contrast, λ-CGNs do not feature 3,6-anhydro bridges and do not make physical gels, but highly viscous solutions [8,10]. These differences may confer biochemical reactivity and mimicry on CGNs in ways not yet determined, since CGN resembles the naturally occurring sulfated glycosaminoglycans (GAGs). In

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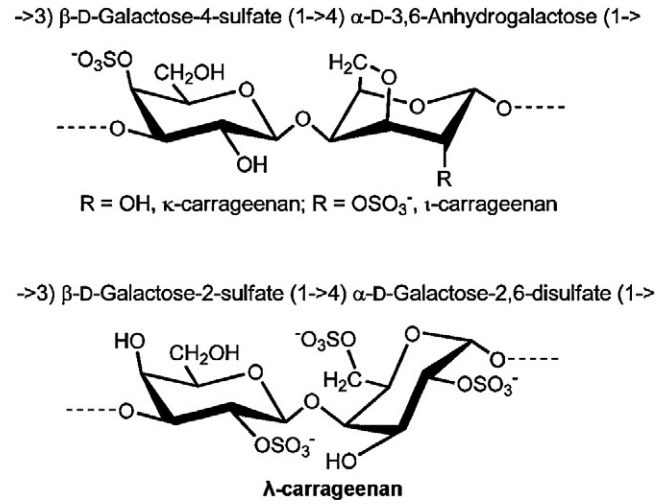


Fig. 1. Sulfated digalactose structure of CGN. The repeating disaccharide structural unit of CGN is demonstrated. β-1→4 and α-1→3 galactosidic linkages connect galactose residues. Sites of sulfation differ among the three major types of CGN.

particular, CGN resembles the sulfated GAGs chondroitin sulfate, dermatan sulfate and keratan sulfate, which also contain sulfated galactose or modified galactose residues. Unlike the naturally occurring GAGs that have β-1→4 and β-1→3 linkages, CGN possesses the unusual α-1→3 galactosidic bond. This structure is recognized as an immune epitope, since it is foreign to humans and large apes, which lack the enzyme α-1,3-galactosyltransferase. The anti-Gal antibody is an antibody that is found universally in human cells and recognizes α-D-Gal-(1→3)-D-Gal linkage [11–15].

CGN exposure predictably induces an increase in interleukin-8 (IL-8) secretion in cells, in tissue culture or in tissues from human or animal colon [16–18]. Mechanisms of CGN-induced IL-8 activation require nuclear localization of nuclear factor κB (NFκB) and proceed by at least two different pathways: a toll-like receptor 4 (TLR4)–B-cell lymphoma/leukemia 10 (BCL10)-mediated pathway and a reactive oxygen species (ROS)-mediated pathway [17,18]. These pathways appear to activate different components of the Iκ B kinase (IKK) signalosome, influencing NFκB by effects predominantly on either IKKβ or IKKγ [19,20].

As we elucidate the biological effects of CGN, one of the essential considerations is how modification of specific structural features of CGN impacts on its biological reactivity. To evaluate the relationship between specific structural features of CGN and cellular responses, we performed experiments to determine how degradation of CGN by galactosidases and carrageenases (CGNases) alters the CGN-induced stimulation of IL-8 and BCL10. We measured IL-8 secretion and BCL10 production following pretreatment of CGNs by these enzymes and documented changes in CGN by gel electrophoresis to better understand how the specific chemical structure of CGN contributes to inflammatory response. We present these findings in this report.

2. Materials and methods

2.1. Cell culture

The NCM460 cell line is a human colonic mucosal epithelial cell line that is nontransfected and nonmalignant. It was originally derived from the normal colon mucosa of a 68-year-old Hispanic male [21]. Cells used in these experiments were grown in M3:10 medium (INCELL, San Antonio, TX) and maintained at 37°C in a humidified 5% CO_2 environment, with changes of medium twice weekly. Confluent cells in T-25 flasks were harvested by EDTA trypsin and subcultured in 6-well or 12-well tissue culture plates. Cells were treated for 24 h with λ-CGN, κ-CGN, $κ_8$-CGN (eight-disaccharide fragment of κ-CGN) [22] or ι-CGN at a concentration of 1 mg/L, either without predigestion or predigested ×24 h with CGNase or galactosidase, singly or in combination. At the end of the treatment, spent media were collected from control and treated wells and stored at −80°C until further analysis. Cells were harvested by scraping, and total cell protein was measured by BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard.

TLR4-blocking antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 20 mg/L was added to some of the NCM460 cell preparations for 24 h, while incubated at 37°C in a humidified incubator with 5% CO_2 , prior to treatment with κ-CGN predigested by enzymes (see the text below).

2.2. Digestion of κ-CGN and ι-CGN

κ-CGNase and ι-CGNase were gifts from Dr. Michel Gurvan [23–25]. CGNases, which cleave the β-1→4 linkages of β-1→4-α-1→3 galactans, were used at a concentration of 3 mg/L to digest 1 mg/L CGN for 24 h prior to exposure of NCM460 cells. κ-CGNase was prepared at a concentration of 2 g/L in a buffer composed of 50 mM Tris–HCl (pH 7.5) and 100 mM NaCl. ι-CGNase was used at a concentration of 0.6 g/L in a buffer composed of 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM $CaCl_2$ and 5% glycerol. Enzymes were stored at 4°C. CGNase at a concentration of 3 mg/L was used to digest 1 mg of CGN. Subsequently, cells were treated with the digested CGN at a concentration of 1 mg/L in a 5% CO_2 environment for 24 h (unless stated otherwise).

The galactosidases — recombinant α-1→(3,6)-galactosidase from *Escherichia coli* (10–200 U/L; Calbiochem EMD Chemicals, Inc., Gibbstown, NJ), β-1→(3,6)-galactosidase (20 U/L; Calbiochem), α-1→6-galactosidase (20 U/L; Sigma Chemical Co., St. Louis, MO) and β-1→4-galactosidase (6–20 U/L; Sigma) — were obtained and combined with CGN for predigestion at 37°C in a 5% CO_2 environment for 24 h, and then the mixture was added to NCM460 cells grown in microwells of 12-well tissue culture plates.

κ-CGN, $κ_8$-CGN and ι-CGN were digested with galactosidase and/or CGNase, either alone or in combination. Control wells received only the enzymes incubated under similar conditions for 24 h. After 24 h of exposure to the CGN–enzyme mixture, spent media were collected and assayed for IL-8, and cells were harvested, lysed and assayed for either total cell protein or BCL10.

2.3. ELISA for IL-8

The secretion of IL-8 in the spent media of control and treated NCM460 cells was determined by the DuoSet ELISA kit for human IL-8 (R&D Systems, Minneapolis, MN), as previously reported [16]. Microtiter plates were precoated with specific anti-IL-8 monoclonal antibody to capture IL-8 in the spent media, and IL-8 bound to the antibody-coated wells of the microtiter plate was detected by biotin-conjugated secondary IL-8 antibody and streptavidin–horseradish peroxidase (HRP). Hydrogen peroxide–tetramethylbenzidine chromogenic substrate was used to develop color and to measure bound HRP. Color intensity was read at 450 nm, with a reference filter of 570 nm in an ELISA plate reader (SLT; Spectra), and was proportional to HRP activity and IL-8 concentration in the particular sample. IL-8 concentrations were extrapolated from a standard curve plotted by using known concentrations of IL-8. Sample values were normalized with total protein content (BCA protein assay kit; Pierce) and expressed as picograms per milligram (or ng/g) of cellular protein.

2.4. ELISA for BCL10

BCL10 in control and treated NCM460 cells was determined by solid-phase sandwich ELISA, the development of which has been previously reported [26]. Control and treated cells were lysed in RIPA buffer (50 mM Tris–HCl containing 0.15 M NaCl, 1% Nonidet P40, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulfate, pH 7.4), and cell extracts were stored at −80°C until assayed. BCL10 molecules in samples or standards were captured in the wells of a microtiter plate precoated with rabbit polyclonal antibody to BCL10 (QED Bioscience, San Diego, CA). Immobilized BCL10 molecules were detected by a mouse monoclonal antibody to BCL10 (Novus Biologicals, Littleton, CO) and by goat anti-mouse IgG–HRP complex (Santa Cruz Biotechnology). The peroxidase enzyme activity bound to BCL10 molecules was determined by a chromogenic reaction with hydrogen peroxide–tetramethylbenzidine. Color development due to enzymatic activity was stopped by 2 N sulfuric acid, and color intensity was measured at 450 nm in an ELISA plate reader (SLT; Spectra). BCL10 concentrations were extrapolated using a standard curve derived from known concentrations of recombinant BCL10 (Calbiochem EMD Bioscience, San Diego, CA). Sample values were normalized with the total cell protein concentrations determined by BCA protein assay kit (Pierce).

2.5. Polyacrylamide gel electrophoresis following digestion of κ-CGN by galactosidases and/or CGNase

Heparin oligosaccharide standards and κ-CGN (100 mg/L) samples were run on 15% polyacrylamide gel electrophoresis (PAGE) and stained with alcian blue. The five samples were as follows: undigested κ-CGN, κ-CGN treated with α-1→(3,6)-galactosidase (20 U/L), κ-CGN treated with α-1→6-galactosidase (20 U/L), κ-CGN

Table 1
IL-8 secreted by NCM460 cells in response to ι-CGN, κ-CGN and short κ-CGN following pretreatment by CGNase or galactosidase

Exposure[a]	IL-8 (pg/mg cell protein)
Control	616 (17)
λ-CGN	1273 (30)
ι-CGN (4-h exposure)	924 (47)
ι-CGN	950 (48)
ι-CGNase	748 (16)
ι-CGN (4-h exposure)+ι-CGNase	1375 (88)
ι-CGN+ι-CGNase	1509 (91)
ι-CGN+α-1→(3,6)-galactosidase (20 U/L)	795 (27)
ι-CGN+ι-CGNase+α-1→(3,6)-Gal (20 U/L)	1340 (92)
κ-CGN	1011 (52)
κ-CGN (3 mg/L)	1060 (42)
κ-CGN+β-1→4-galactosidase	1016 (70)
κ-CGN+α-1→6-galactosidase	1014 (17)
κ-CGN+α-1→6-galactosidase+β-1→4-galactosidase	1020 (64)
κ-CGN+α-1→(3,6)-galactosidase (20 U/L)	896 (22)
κ-CGN+α-1→(3,6)-galactosidase (50 U/L)	782 (27)
κ-CGN+α-1→(3,6)-galactosidase (100 U/L)	698 (23)
κ-CGN+α-1→(3,6)-galactosidase (200 U/L)	676 (50)
κ-CGN+α-1→(3,6)-galactosidase (20 U/L)+β-1→4-galactosidase	891 (4)
κ-CGN+κ-CGNase	1623 (54)
κ-CGN+κ-CGNase+β-1→4-galactosidase	1629 (33)
κ-CGN+α-1→(3,6)-galactosidase (20 U/L)+κ-CGNase	1431 (15)
κ-CGN+α-1→(3,6)-galactosidase (50 U/L)+κ-CGNase	1245 (57)
κ-CGN+α-1→(3,6)-galactosidase (100 U/L)+κ-CGNase	1105 (22)
κ-CGN (3 mg/L) ~1500 mol wt	1596 (72)
κ-CGN (3 mg/L) ~4000 mol wt	1737 (63)
κ-CGN (3 mg/L) ~8000 mol wt	1605 (41)
κ-CGN8	1712 (68)
κ-CGN8+β-1→4-galactosidase	1744 (121)
κ-CGN8+α-1→6-galactosidase	1715 (74)
κ-CGN8+α-1→6-galactosidase+β-1→4-galactosidase	1732 (121)
κ-CGN8+α-1→(3,6)-galactosidase (20 U/L)	1408 (41)
κ-CGN8 α-1→(3,6)-galactosidase (20 U/L)+β-1→4-galactosidase	1399 (49)
κ-CGN8+κ-CGNase	1822 (58)
κ-CGN8+κ-CGNase+α-1→(3,6)-galactosidase (20 U/L)	1514 (38)

Data are presented as the mean (S.D.) of at least three biological replicates, with technical duplicates of each determination. Enzyme concentrations are expressed as units per liter, based on their established biological reactivity with their standard substrate.

[a] Unless stated otherwise, the concentration of CGN was 1 mg/L and exposure was 24 h.

treated with κ-CGNase (3 mg/L), and κ-CGN treated with κ-CGNase (3 mg/L) and α-1→(3,6)-galactosidase (20 U/L).

2.6. Measurement of ROS

Production of ROS by DSS was measured by hydroethidine fluorescence [27]. Cells were grown in a 96-well cell culture plate. After 24 h, the medium was changed, and treatment with κ-CGN (1 mg/L ×24 h) or κ-CGN predigested ×24 h with α-1→(3,6)-galactosidase (20 and 100 U/L) or κ-CGNase (3 mg/L), separately and in combination, was started. At the end of the treatment period, the medium in each well was removed, cells were washed with Hank's balanced salt solution (HBSS) and cells were covered with 200 μl of HBSS containing 10 μM dihydroethidine (Sigma). Cultures were incubated for 1 h at 37°C and 5% CO_2, then the medium was removed and replaced with fresh HBSS (200 μl/well). Intracellular HE fluorescence was measured using a microplate fluorescence reader (FL600; Bio-Tek Instruments, Inc., Winooshi, VT) at an excitation wavelength of 488 nm with a 610-nm emission filter.

2.7. Statistical analysis

Data are presented as the mean±standard deviation (S.D.) of at least three biological replicates and two technical duplicates of each determination, unless stated otherwise. Statistical significance was determined by one-way ANOVA with Tukey–Kramer posttest for multiple comparisons using Instat software, unless stated otherwise. Two-tailed *t* test, performed using Microsoft Excel software, was used for some comparisons. $P \leq .05$ is considered statistically significant. The correlation coefficient of BCL10 and IL-8 values following exposure to α-1→(3,6)-galactosidase of different concentrations was calculated by linear regression using Excel software, and the linear trend of BCL10 and IL-8 with increasing concentrations of α-1→(3,6)-galactosidase was calculated using Instat software (*$P \leq .05$; **$P \leq .01$ and ***$P \leq .001$ in the figures).

3. Results

3.1. IL-8 production by different CGNs

In a series of experiments testing different forms of CGN — including λ, κ and ι (Fig. 1) of high molecular weight (>1×10^6 g/mol mol wt) and degraded forms of κ-CGN of lower molecular weight — we have detected differences in the CGN-induced secretion of IL-8 by NCM460 cells in culture. These results are presented in Table 1.

IL-8 production by κ-CGNs of different disaccharide chain lengths, including 3, 8 and 16 disaccharide pairs (molecular weights of ~1500, ~4000 and ~8000), was compared. The shorter degraded CGNs induced a higher IL-8 response, consistent with increased exposure of the α-1→3-epitope.

λ-CGN produced an IL-8 response higher than that produced by κ-CGN ($P<.01$) or ι-CGN ($P<.001$), perhaps attributable to a more favorable structure with less internal bonding between C3 and C6 of the nonsulfated saccharide, producing the pyranose epimer.

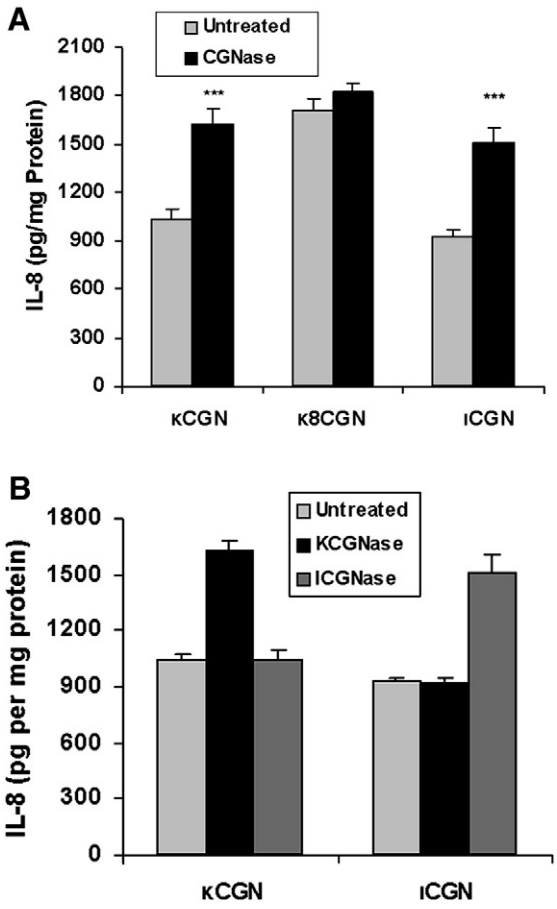


Fig. 2. Increased IL-8 response to CGN following treatment with CGNase. (A) NCM460 cells were exposed to either κ-CGN, short κ_8-CGN or ι-CGN that had been pretreated with κ-CGNase or ι-CGNase (3 mg/L) for 24 h. Significant increases in IL-8 production occurred for κ-CGN and ι-CGN ($P<.001$, paired *t* test), but not for the short κ-CGN comprising eight disaccharides. These changes suggest that digestion with CGNase led to increased exposure of the immunogenic epitope in undegraded CGNs and that the impact of digestion by CGNase was less in the already degraded short κ-CGN. Data are presented as mean±S.D. and are representative of six independent biological experiments, with technical duplicates of each determination. (B) Pretreatment of κ-CGN with ι-CGNase or pretreatment of ι-CGN with κ-CGNase produced no changes in IL-8 responses, consistent with the specificity of the CGNases for specific forms of CGN. Data are presented as the mean±S.D. of three independent biological experiments, with technical replicates of each determination.

3.2. CGNases increase IL-8 response

CGN (1 mg/L) was pretreated for 24 h with purified κ-CGNase or ι-CGNase (3 mg/L) — enzymes highly specified for cleavage of the β-1→4 linkages of β-1→4-α-1→3 galactans that are present in κ-CGN or λ-CGN. NCM460 cells were treated with these preparations for 24 h, and the secreted IL-8 was measured. Following treatment with CGNase, IL-8 secretion increased significantly (Fig. 2A; $P<.001$, paired t test). In the short degraded κ-CGN composed of eight disaccharides, baseline IL-8 secretion was higher, and the increase in IL-8 following treatment with CGNase was less than that following treatment with the undegraded κ-CGN. These differences suggest that there was greater exposure of the cells to the immune epitope in the degraded CGN at baseline, and that there was less of an increase in interaction between the immune epitope and the TLR4 following CGNase treatment of the already degraded CGN. The impact of the CGNases was specific to the κ form or the λ form, with no evidence of cross-reactivity (Fig. 2B).

3.3. Only α-1→(3,6)-galactosidase has an effect on IL-8 secretion

NCM460 cells were treated for 24 h with κ-CGN or ι-CGN (1 mg/L) that had been combined with one of four different galactosidases: either α-1→6-galactosidase (20 U/L), β-1→4-galactosidase (20 U/L), β-1→(3,6)-galactosidase (20 U/L) or α-1→(3,6)-galactosidase for 24 h (20 U/L). Results demonstrated no effect of either α-1→6-galactosidase (Fig. 3A), β-1→4-galactosidase (Fig. 3B) or β-1→(3,6)-galactosidase on IL-8 response (data not shown), in contrast to significant declines in association with exposure to α-1→(3,6)-galactosidase (Fig. 3C). A dose–response effect was evident between the concentration of α-1→(3,6)-galactosidase and the concentration of the secreted IL-8 (Fig. 3D) ($P<.001$, one-way ANOVA with Tukey–Kramer posttest).

3.4. Effect of combined α-1→(3,6)-galactosidase and CGNase

NCM460 cells were exposed to CGNs that were pretreated with a combination of κ-CGNase (3 mg/L) and α-1→(3,6)-galactosidase (20 U/L) (Fig. 4A). At this concentration of α-1→(3,6)-galactosidase, IL-8 secretion rose significantly following exposure to undegraded κ-CGNs and ι-CGNs, and declined following exposure to degraded κ_8-CGN. As the concentration of α-1→(3,6)-galactosidase increased from 20 to 100 U/L, the increase in the IL-8 secretion induced by the exposure of κ-CGN to CGNase declined significantly (Fig. 4B; $P<.001$, one-way ANOVA with Tukey–Kramer posttest). These changes were

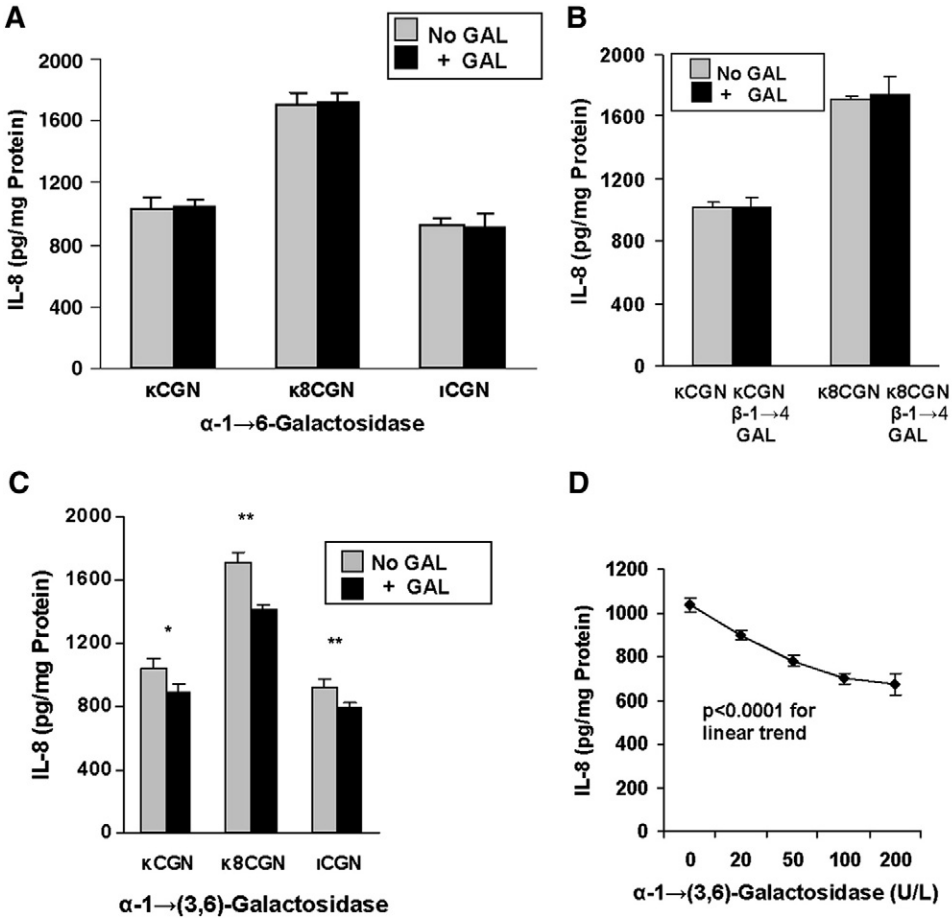


Fig. 3. Only galactosidase that hydrolyzed the α-1→3-galactosidic bond produced a decline in IL-8 production. (A) Pretreatment of CGNs by α-1→6-galactosidase (20 U/L) had no effect on IL-8 secretion in NCM460 cells. Data are presented as mean±S.D. and are representative of nine independent biological experiments, with technical replicates of each determination. (B) Pretreatment by β-1→4-galactosidase (20 U/L) of κ-CGN and short κ_8-CGN had no effect on IL-8 secretion in NCM460 cells. Data are presented as mean±S.D. and are representative of nine independent biological experiments, with technical replicates of each determination. Gal, galactosidase. (C) Pretreatment of CGNs by α-1→(3,6)-galactosidase (20 U/L) produced significant declines ($P=.03$ for κ-CGN; $P=.003$ for κ_8-CGN; $P=.008$ for ι-CGN; paired t test) in IL-8 secretion in NCM460 cells, indicating the importance of the Gal-α-(1→3)-Gal epitope for the CGN-induced IL-8 response. Data are presented as mean±S.D. and are representative of 14 independent biological experiments, with technical duplicates for κ-CGN and $n=6$ for short κ_8-CGN and ι-CGN. (D) IL-8 secretion decreased following exposure to increasing concentrations of α-1→(3,6)-galactosidase (20–200 U/L) in NCM460 cells ($P<.001$, one-way ANOVA with Tukey–Kramer posttest; $P<.0001$ for linear trend). Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination.

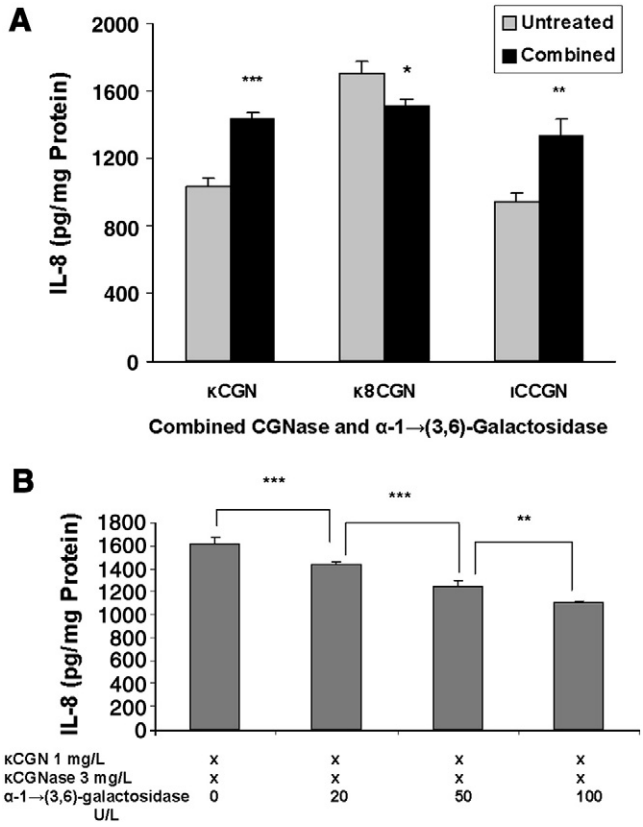


Fig. 4. Effect of combined exposure to CGNase and α-1→(3,6)-galactosidase. (A) Specific κ-CGNase or ι-CGNase (3 mg/L) and α-1→(3,6)-galactosidase (20 U/L) were combined to pretreat CGN for 24 h. IL-8 secretion increased when NCM460 cells were exposed to undegraded κ-CGN or ι-CGN, reflecting a predominant effect of the CGNase. In contrast, in the short degraded κ-CGN ($κ_8$-CGN), the combination resulted in a decline in IL-8 secretion, indicating an increased effect of the α-1→(3,6)-galactosidase. Differences in IL-8 are statistically significant (P=.0004 for κ-CGN; P=.02 for $κ_8$-CGN; P=.003 for ι-CGN; paired t test). Data are presented as the mean ±S.D. of nine independent biological experiments, with technical duplicates of each determination for κ-CGN and n=6 for short $κ_8$-CGN and ι-CGN. (B) Dose response to α-1→(3,6)-galactosidase was evident, since secreted IL-8 declined with exposure of undegraded κ-CGN to increasing concentrations of α-1→(3,6)-galactosidase, including 20, 50 and 100 U/L in the presence of κ-CGNase (3 mg/L) (P<.001 for differences between 0 and 20 U/L, and between 20 and 50 U/L; P<.01 for differences between 50 and 100 U/L; one-way ANOVA with Tukey–Kramer posttest). Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination.

consistent with altered exposure to α-Gal-(1→3)-Gal configuration by enzymatic digestion of the CGN.

3.5. PAGE of κ-CGN digestion products

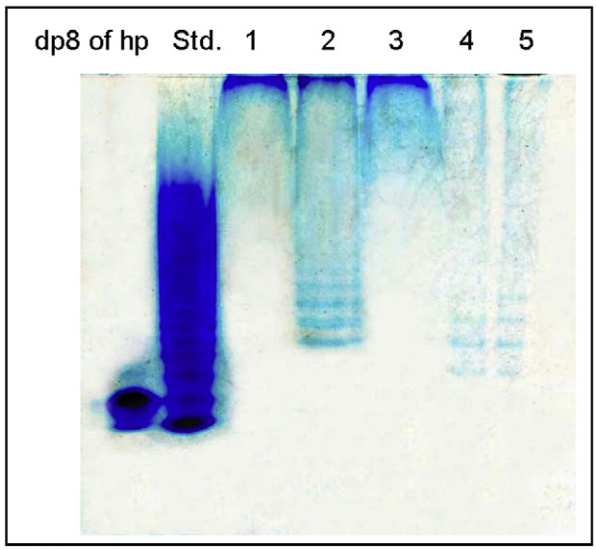
Samples of κ-CGN were exposed to galactosidases and/or κ-CGNase for 24 h, and digestion products were analyzed by PAGE. Undegraded oligosaccharides were present in Samples 1 and 3, representing undegraded and untreated κ-CGN and κ-CGN exposed to α-1→6-galactosidase, respectively (Fig. 5). Lower-molecular-weight oligosaccharides were observed in Samples 2, 4 and 5, indicating degradation by enzymatic treatment. Sample 2, which was exposed to α-1→(3,6)-galactosidase, had polysaccharide remaining at the top of the gel, indicating incomplete degradation. In contrast, in Samples 4 and 5, only a small amount of polysaccharide remained at the top of the gel, consistent with a much more complete degradation than in Sample 2. Lower-molecular-weight products were present in Samples 4 and 5 than in Sample 2. Sample 3 was resistant to degradation by the α-1→6-galactosidase.

3.6. Effect of enzymes on BCL10 production

When NCM460 cells were exposed to CGN in the presence of κ-CGNase (3 mg/L) (Fig. 6A), significant increases in BCL10 occurred, consistent with an increase in immune stimulation when CGN was digested by CGNase. In contrast, pretreatment with α-1→(3,6)-galactosidase (20 U/L) produced marked declines in BCL10 response (Fig. 6B), consistent with reduced exposure to the immunogenic epitope. Following pretreatment with α-1→6-galactosidase (20 U/L; Fig. 6C), β-1→4-galactosidase (20 U/L; Fig. 6D) or β-1→(3,6)-galactosidase (data not shown), no changes in the BCL10 protein occurred, indicating no effect of these enzymes on the immune reactivity of CGN. When κ-CGNase and α-1→(3,6)-galactosidase (20 U/L) were combined, the increases in BCL10 produced by κ-CGNase alone were reduced, and BCL10 content was less in degraded $κ_8$-CGN-exposed cells than when untreated (Fig. 6E). The κ-CGN-induced increase in BCL10 declined with increasing concentrations of α-1→(3,6)-galactosidase (Fig. 6F; P<.001, one-way ANOVA with Tukey–Kramer posttest). The declines in κ-CGN-induced increases in IL-8 and BCL10 with exposure to increasing concentrations of α-1→(3,6)-galactosidase were compared by linear regression and found to be highly correlated (r=.9949), indicating the close association between declines in BCL10 and IL-8 and suggesting a mechanistic link.

3.7. No effect of enzymes on ROS production

The baseline ROS production in NCM460 cells was 912±67, as measured by hydroethidine fluorescence. Following exposure to κ-CGN for 24 h, ROS production increased to 8509±367. No significant



Std=heparin;
1 = κCGN 100 g/L;
2 = κCGN 100 g/L + α-1→(3,6)-galactosidase (10 U/L);
3 = κCGN 100 g/L + α-1→6-galactosidase (20 U/L);
4 = κCGN 100 g/L + κ-CGNase (3 g/L);
5 = κCGN 100 g/L + α-1→(3,6)-galactosidase (10 U/L) +κCGNase (3 g/L)

Fig. 5. Demonstration of κ-CGN degradation products following enzymatic digestion by PAGE. Digestion products of heparin are presented as molecular weight standards (Std). Lane 1, high-molecular-weight κ-CGN without exposure to enzymes. Lane 2, κ-CGN digested by α-1→(3,6)-galactosidase. Lane 3, κ-CGN undigested by α-1→6-galactosidase. Lane 4, κ-CGN digested by κ-CGNase. Lane 5, κ-CGN digested by a combination of κ-CGNase and α-1→(3,6)-galactosidase. Lower-molecular-weight products are shown in Lanes 4 and 5 than in Lane 2. In experiments with NCM460 cells, κ-CGNase was used at a 10-fold lower concentration relative to the amount of CGN in this analysis.

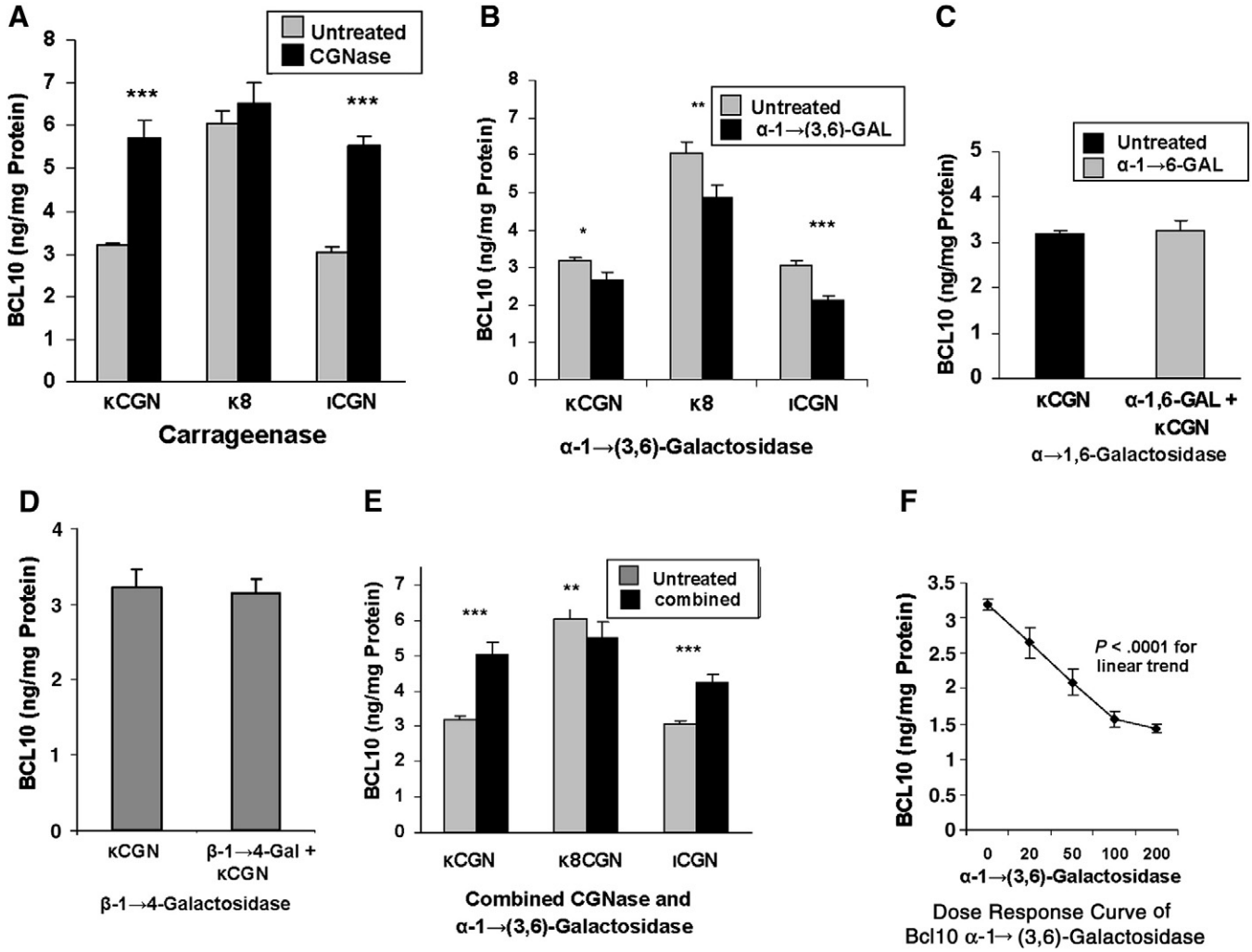


Fig. 6. Cellular BCL10 production is modified by exposure to CGN-metabolizing enzymes. (A) BCL10 production in NCM460 cells increased following pretreatment of κ-CGN, short degraded $κ_8$-CGN and ι-CGN (1 mg/L) with specific CGNase (3 mg/L), consistent with increased activation of a BCL10-mediated pathway following increased exposure to the α-Gal-(1→3)-Gal epitope. Results were statistically significant for κ-CGNs and ι-CGNs (P<.001, paired t test). Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination. (B) Following exposure to α-1→(3,6)-galactosidase (20 U/L), BCL10 values declined, reflecting less exposure of the immunogenic epitope. Results were statistically significant (P=.02 for κ-CGN; P=.01 for $κ_8$-CGN; P=.0009 for ι-CGN; paired t test). Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination. (C) Following exposure to α-1→,6-galactosidase (20 U/L), no change in BCL10 content was found. Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination. (D) Following exposure to (20 U/L), no change in BCL10 content occurred. Data are presented as the mean±S.D. β-1→4-galactosidase of three independent biological experiments, with technical duplicates of each determination. (E) When NCM460 cells were exposed to CGN pretreated with both specific CGNase (3 mg/L) and α-1→(3,6)-galactosidase (20 U/L), cellular BCL10 content increased following exposure to κ-CGN and ι-CGN, but declined following exposure to the short degraded κ-CGN, in a pattern similar to that observed with IL-8. Results are statistically significant, with increases for undegraded κ-CGN and ι-CGN (P<.001, paired t test) and decline for $κ_8$-CGN (P=.01, paired t test). Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination. (F) With increasing concentrations of α-1→(3,6)-galactosidase (20, 50 and 100 U/L), increasing declines in BCL10 production were observed in NCM460 cells, again similar to the changes observed in IL-8 (P<.001, one-way ANOVA with Tukey–Kramer posttest; P<.0001 for linear trend; r^2=.94 for linear trend). Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination.

changes in ROS production were observed when κ-CGN was pretreated with either κ-CGNase (3 mg/L), α-1→(3,6)-galactosidase (20 U/L) or these enzymes in combination. Previously, we have demonstrated that CGN induced increases in IL-8 by a TLR4–BCL10 immune-mediated pathway and an ROS-mediated pathway. The lack of change in ROS following exposure to CGNase or α-1→(3,6)-galactosidase is consistent with a pathway of immune activation by CGN that is initiated by the α-Gal-(1→3)-Gal epitope.

3.8. TLR4-blocking antibody inhibits the impact of enzymes on IL-8 secretion

When NCM460 cells were exposed to TLR4-blocking antibody for 24 h prior to treatment with κ-CGN predigested by κ-CGNase or α-1→(3,6)-galactosidase, the previously detected changes in the secretion of IL-8 were inhibited (Fig. 7). These results confirm that the modified CGN acts through the TLR4–BCL10-mediated pathway.

4. Discussion

For decades, CGN has been used in food products to improve the texture of processed foods and to increase the solubility of other ingredients, such as casein found in milk products [28]. In the laboratory, CGN has also been used for decades to induce inflammation and to study the mediators of inflammation and the effectiveness of anti-inflammatory treatments [29–32]. Our recent work has identified two mechanisms by which CGN predictably induces inflammation in colonic epithelial cells [16–18]. The first mechanism

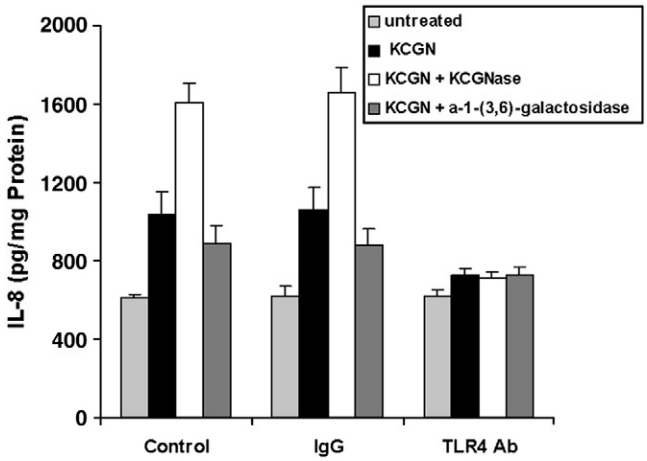


Fig. 7. TLR4-blocking antibody inhibits changes in IL-8 secretion caused by enzymes. When NCM460 cells were exposed to TLR4-blocking antibody for 24 h prior to treatment by κ-CGN (1 mg/L) that had been digested by κ-CGNase or α-1→(3,6)-galactosidase, the previously demonstrated changes in IL-8 secretion were inhibited. These findings support the interpretation that these enzymes affect the interaction of CGN with TLR4. Data are presented as the mean±S.D. of three independent biological experiments, with technical duplicates of each determination.

involves the cell surface receptor TLR4 and includes MyD88, IRAK and BCL10. BCL10 has been recognized as an important mediator of inflammation in immune cells due to its association with IKKγ (also called NEMO), which is the regulatory subunit of the IKK signalosome. The activation of BCL10 appears to influence the ubiquitination of IKKγ, enabling the phosphorylation of IκBα by the catalytic components (IKKβ and IKKα) of the IKK complex [19,20]. This permits exposure of the nuclear localization signal of NFκB, leading to increased production of IL-8. In MALT lymphomas, translocations involving BCL10 are associated with constitutive activation of BCL10 [33,34]. Hence, the finding that specific enzymatic exposures to CGN alter the production of BCL10 and IL-8 provides a mechanistic insight into a basic pathway of immune activation through the α-Gal-(1→3)-Gal epitope. Variation in BCL10 responsiveness through enzymatic modification of galactosyl bonds appears to signal whether or not there is activation of an immune-mediated mechanism of inflammation. The presence of the 3,6-anhydro bridge in D-galactose in CGN is also unique to red algae and is absent in humans, suggesting the possible involvement of this conformation in immune response as well. The action of CGNases releases CGN oligosaccharides from κ-CGN with 3,6-anhydro-D-galactose as the nonreducing end, and from ι-CGN with 3,6-andydro-D-galactose-2-sulfate residues as the nonreducing end. The immune response may therefore be activated by the presence of the 3,6-anhydro-D-Gal-α-(1,3)-D-Gal-4-sulfate epitope of κ-CGN or by the presence of the 3,6-anhydro-D-galactose-2-sulfate-α-(1,3)-D-Gal-4-sulfate epitope of ι-CGN, in contrast to λ-CGN, in which the 3,6-anhydro linkage does not form. The IL-8 response to λ-CGN is significantly greater than the IL-8 response to either ι-CGN or κ-CGN, perhaps attributable to increased exposure of the α-Gal-(1→3)-Gal epitope. Also, the IL-8 response to ι-CGN is always significantly less than the IL-8 response to either κ-CGN or λ-CGN; this difference may be attributable to the presence of ester sulfate at C2 of the 3,6-anhydro-D-galactose unit, reducing immune reactivity to some extent.

A second mechanism by which CGN activates an inflammatory cascade is mediated through ROS, leading to a decline in the phosphorylation of Hsp27, which is involved in reciprocal phosphorylation with IKKβ by protein phosphatase 2A [18]. The study findings demonstrate that changes in ROS production are not responsible for the variation in IL-8 found after treatment of CGN with α-1→(3,6)-galactosidase or CGNase. The additional effects of CGN that we have reported in colonic epithelial cells include changes in the bone morphogenic protein 4/Wnt9a axis and in cell cycle arrest [35,36]. These may or may not be mediated independently of inflammatory cascades. We have not yet evaluated the impact of exposure to galactosidases and CGNases on other responses to CGN. Other effects may arise from other structural features of CGN, including mimicry of naturally occurring GAGs.

Since variation in the bacterial composition of the colonic microflora may be associated with variation in the types of galactosidases and the presence or the absence of CGNases, the metabolism of CGN may vary from individual to individual or over time in the same individual. The propensity for the development of intestinal inflammation from exposure to CGN may be related to the colonic subpopulation of specific organisms that produce these enzymes. Differences in the colonic microflora may contribute to the development of inflammatory bowel disease, colonic polyps or colonic neoplasms, making some individuals more or less susceptible to harmful sequelae from exposure to dietary CGN.

It is pertinent to note that the α-Gal(1→3)Gal epitope is present in some bacterial lipopolysaccharides (LPS) that are known to be toxic. These include two serogroups of *E. coli* — 020 and 086 — that express LPS with the α-D-Gal-(1→3)-D-gal epitope [37–39]. These have been associated with pathogenicity; 020 cross-reacts with *Klebsiella pneumoniae* 04, and 086 cross-reacts with *Salmonella* 043. The overlap between CGN and epitopes of these pathogenic bacteria demonstrates that this specific configuration found in CGN is likely to evoke immune responses to CGN that are manifested by increased BCL10 and IL-8. The α-Gal-(1→3)-Gal epitope has also been associated with cetuximab-induced anaphylaxis involving an IgE response [40]. This epitope resembles to some extent the epitope of the B-blood group antigen, but lacks the associated fucose residue. A different α-galactosyltransferase enzyme is required to generate the α-Gal-(1→3)-Gal epitope and produces a different configuration.

Additional experiments are required to determine whether the α-Gal-(1→3)-Gal structure directly interacts with TLR4, or whether MD-2, CD14 or LBP is required to properly position the CGN, as occurs with LPS. Leucine-rich regions within the extracellular domain (ECD) of TLR4 may provide the backbone for direct interaction with CGN, but a specific structural conformation within the ECD that recognizes the α-1,3-galactosidic bond has not yet been identified. Determination of the crystal structures of TLR4 and MD-2 with eritoran showed that LPS required interaction with MD-2 in order to bind with TLR4 [41]. Phe126 and His155 residues of MD-2 were required for LPS-induced dimerization of the TLR4–MD-2 mouse complex. Subsequent experiments will help to elucidate whether or not MD-2 is required for the CGN-induced activation of the TLR4-mediated pathway.

The study findings suggest that treatment of CGN by an enzyme with α-1→3-galactosidase activity may lead to a reduced inflammatory response to CGN. These results have the potential to ameliorate the harmful effects of CGN by establishing colonic colonization with bacteria that produce this enzyme. However, since the marked biological reactivity of CGN may arise from more than this epitope, reduction of human exposure to CGN remains a more reliable means to reduce the harmful effects of CGN.

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