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Barbara Zeitlin Kravets CCN LDN,
Licensed Dietitian Nutritionist,
Certified Clinical Nutritionist,
Medical Nutrition Therapy,
Phone 1 847 870 9514, Fax 1 847 239 6724,

Also send to my other email licnutrition@msn.com
Web site www.findanutritionist.com/practitioners/licnutrition
Editor of The NutritionNews ©, internationally distributed, cutting edge, peer reviewed, journal abstract, email nutrition newsletter

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<http://www.rxlist.com/stromectol-drug.htm>

STROMECTOL

(Ivermectin) is a semisynthetic, anthelmintic agent for oral administration. Ivermectin is derived from the avermectins, a class of highly active broad-spectrum, anti-parasitic agents isolated from the fermentation products of Streptomyces avermitilis. Ivermectin is a mixture containing at least 90% 5-odemethyl-

22,23-dihydroavermectin A1a and less than 10% 5-O-demethyl-25-de(1-methylpropyl)-22,23-dihydro-25-(1-methylethyl)avermectin A1a, generally referred to as 22,23-dihydroavermectin B1a and B1b, or H2B1a and H2B1b, respectively. The respective empirical formulas are C₄₈H₇₄O₁₄ and C₄₇H₇₂O₁₄, with molecular weights of 875.10 and 861.07, respectively. The structural formulas are:

Component B1a, R = C₂H₅ Component B1b, R = CH₃

Ivermectin is a white to yellowish-white, nonhygroscopic, crystalline powder with a melting point of about 155°C. It is insoluble in water but is freely soluble in methanol and soluble in 95% ethanol.

STROMEKTOL is available in 3-mg tablets containing the following inactive ingredients: microcrystalline cellulose, pregelatinized starch, magnesium stearate, butylated hydroxyanisole, and citric acid powder (anhydrous).

Last updated on RxList: 5/26/2009

STROMEKTOL is indicated for the treatment of the following infections:

Strongyloidiasis of the intestinal tract. STROMEKTOL is indicated for the treatment of intestinal (i.e., nondisseminated) strongyloidiasis due to the nematode parasite *Strongyloides stercoralis*.

This indication is based on clinical studies of both comparative and open-label designs, in which 64-100% of infected patients were cured following a single 200-mcg/kg dose of ivermectin. (See CLINICAL PHARMACOLOGY, Clinical Studies.)

Onchocerciasis. STROMEKTOL is indicated for the treatment of onchocerciasis due to the nematode parasite *Onchocerca volvulus*.

This indication is based on randomized, double-blind, placebo-controlled and comparative studies conducted in 1427 patients in onchocerciasis-endemic areas of West Africa. The comparative studies used diethylcarbamazine citrate (DEC-C).

NOTE: STROMEKTOL has no activity against adult *Onchocerca volvulus* parasites. The adult parasites reside in subcutaneous nodules which are infrequently palpable. Surgical excision of these nodules (nodulectomy) may be considered in the management of patients with onchocerciasis, since this procedure will eliminate the microfilariae-producing adult parasites.

DOSAGE AND ADMINISTRATION

Strongyloidiasis

The recommended dosage of STROMEKTOL for the treatment of strongyloidiasis is a single oral dose designed to provide approximately 200 mcg of ivermectin per kg of body weight. See Table 1 for dosage guidelines. Patients should take tablets on an empty stomach with water. (See CLINICAL PHARMACOLOGY, Pharmacokinetics.) In general, additional doses are not necessary. However, follow-up stool examinations should be performed to verify eradication of infection. (See CLINICAL PHARMACOLOGY, Clinical Studies.)

Table 1 Dosage Guidelines for STROMEKTOL for Strongyloidiasis

Body Weight (kg) Single Oral Dose

Number of 3-mg Tablets

15-24 1 tablet

25-35 2 tablets

36-50 3 tablets

51-65 4 tablets

66-79 5 tablets

≥ 80 200 mcg/kg

Onchocerciasis

The recommended dosage of STROMEKTOL for the treatment of onchocerciasis is a single oral dose designed to provide approximately 150 mcg of ivermectin per kg of body weight. See Table 2 for dosage guidelines. Patients should take tablets on an empty stomach with water. (See CLINICAL PHARMACOLOGY, Pharmacokinetics.) In mass distribution campaigns in international treatment programs, the most commonly used dose interval is 12 months. For the treatment of individual patients, retreatment may be considered at intervals as short as 3 months.

Table 2 Dosage Guidelines for STROMEKTOL for Onchocerciasis

Body Weight (kg) Single Oral Dose

Number of 3-mg Tablets

15-25 1 tablet

26-44 2 tablets

45-64 3 tablets

65-84 4 tablets

≥ 85 150 mcg/kg

Strongyloidiasis

In four clinical studies involving a total of 109 patients given either one or two doses of 170 to 200 mcg/kg of STROMEKTOL, the following adverse reactions were reported as possibly, probably, or definitely related to STROMEKTOL:

Body as a Whole: asthenia/fatigue (0.9%), abdominal pain (0.9%)

Gastrointestinal: anorexia (0.9%), constipation (0.9%), diarrhea (1.8%), nausea (1.8%), vomiting (0.9%)

Nervous System/Psychiatric: dizziness (2.8%), somnolence (0.9%), vertigo (0.9%), tremor (0.9%)

Skin: pruritus (2.8%), rash (0.9%), and urticaria (0.9%).

In comparative trials, patients treated with STROMEKTOL experienced more abdominal distention and chest discomfort than patients treated with albendazole. However, STROMEKTOL was better tolerated than thiabendazole in comparative studies involving 37 patients treated with thiabendazole.

The Mazzotti-type and ophthalmologic reactions associated with the treatment of onchocerciasis or the disease itself would not be expected to occur in strongyloidiasis patients treated with STROMEKTOL. (See ADVERSE REACTIONS, Onchocerciasis.)

Laboratory Test Findings

In clinical trials involving 109 patients given either one or two doses of 170 to 200 mcg/kg STROMEKTOL, the following laboratory abnormalities were seen regardless of drug relationship: elevation in ALT and/or AST (2%), decrease in leukocyte count (3%). Leukopenia and anemia were seen in one patient.

Onchocerciasis

In clinical trials involving 963 adult patients treated with 100 to 200 mcg/kg STROMEKTOL, worsening of the following Mazzotti reactions during the first 4 days post-treatment were reported: arthralgia/synovitis (9.3%), axillary lymph node enlargement and tenderness (11.0% and 4.4%, respectively), cervical lymph node enlargement and tenderness (5.3% and 1.2%, respectively), inguinal lymph node enlargement and tenderness (12.6% and 13.9%, respectively), other lymph node enlargement and tenderness (3.0% and 1.9%, respectively), pruritus (27.5%), skin involvement including edema, papular and pustular or frank urticarial rash (22.7%), and fever (22.6%). (See WARNINGS.)

In clinical trials, ophthalmological conditions were examined in 963 adult patients before treatment, at day 3, and months 3 and 6 after treatment with 100 to 200 mcg/kg STROMEKTOL. Changes observed were primarily deterioration from baseline 3 days post-treatment. Most changes either returned to baseline condition or improved over baseline severity at the month 3 and 6 visits. The percentages of patients with worsening of the following conditions at day 3, month 3 and 6, respectively, were: limbitis: 5.5%, 4.8%, and 3.5% and punctate opacity: 1.8%, 1.8%, and 1.4%. The corresponding percentages for patients treated with placebo were: limbitis: 6.2%, 9.9%, and 9.4% and punctate opacity: 2.0%, 6.4%, and 7.2%. (See WARNINGS.)

In clinical trials involving 963 adult patients who received 100 to 200 mcg/kg STROMEKTOL, the following clinical adverse reactions were reported as possibly, probably, or definitely related to the drug in $\geq 1\%$ of the patients: facial edema (1.2%), peripheral edema (3.2%), orthostatic hypotension (1.1%), and tachycardia (3.5%). Drug-related headache and myalgia occurred in $< 1\%$ of patients (0.2% and 0.4%, respectively). However, these were the most common adverse experiences reported overall during these trials regardless of causality (22.3% and 19.7%, respectively).

A similar safety profile was observed in an open study in pediatric patients ages 6 to 13.

The following ophthalmological side effects do occur due to the disease itself but have also been reported after treatment with STROMEKTOL: abnormal sensation in the eyes, eyelid edema, anterior uveitis, conjunctivitis, limbitis, keratitis, and chorioretinitis or choroiditis. These have rarely been severe or associated with loss of vision and have generally resolved without corticosteroid treatment.

Laboratory Test Findings

In controlled clinical trials, the following laboratory adverse experiences were reported as possibly, probably, or definitely related to the drug in $\geq 1\%$ of the patients: eosinophilia (3%) and hemoglobin increase (1%).

Post-Marketing Experience

The following adverse reactions have been reported since the drug was registered overseas:

Onchocerciasis

Conjunctival hemorrhage

All Indications

Hypotension (mainly orthostatic hypotension), worsening of bronchial asthma, toxic epidermal necrolysis, Stevens-Johnson syndrome, seizures, hepatitis, elevation of liver enzymes, and elevation of bilirubin.

DRUG INTERACTIONS

Post-marketing reports of increased INR (International Normalized Ratio) have been rarely reported when ivermectin was co-administered with warfarin.

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WARNINGS

Historical data have shown that microfilaricidal drugs, such as diethylcarbamazine citrate (DEC-C), might cause cutaneous and/or systemic reactions of varying severity (the Mazzotti reaction) and ophthalmological reactions in patients with onchocerciasis. These reactions are probably due to allergic and inflammatory responses to the death of microfilariae. Patients treated with STROMEKTOL for onchocerciasis may experience these reactions in addition to clinical adverse reactions possibly, probably, or definitely related to the drug itself. (See ADVERSE REACTIONS, Onchocerciasis.)

The treatment of severe Mazzotti reactions has not been subjected to controlled clinical trials. Oral hydration, recumbency, intravenous normal saline, and/or parenteral corticosteroids have been used to treat postural hypotension. Antihistamines and/or aspirin have been used for most mild to moderate cases.

PRECAUTIONS

General

After treatment with microfilaricidal drugs, patients with hyperreactive onchodermatitis (sowda) may be more likely than others to experience severe adverse reactions, especially edema and aggravation of onchodermatitis.

Rarely, patients with onchocerciasis who are also heavily infected with *Loa loa* may develop a serious or even fatal encephalopathy either spontaneously or following treatment with an effective microfilaricide. In these patients, the following adverse experiences have also been reported: pain (including neck and back pain), red eye, conjunctival hemorrhage, dyspnea, urinary and/or fecal incontinence, difficulty in standing/walking, mental status changes, confusion, lethargy, stupor, seizures, or coma. This syndrome has been seen very rarely following the use of ivermectin. In individuals who warrant treatment with ivermectin for any reason and have had significant exposure to *Loa loa*-endemic areas of West or Central Africa, pretreatment assessment for loiasis and careful post-treatment follow-up should be implemented.

Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies in animals have not been performed to evaluate the carcinogenic potential of ivermectin. Ivermectin was not genotoxic in vitro in the Ames microbial mutagenicity assay of *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 with and without rat liver enzyme activation, the Mouse Lymphoma Cell Line L5178Y (cytotoxicity and mutagenicity) assays, or the unscheduled DNA synthesis assay in human fibroblasts.

Ivermectin had no adverse effects on the fertility in rats in studies at repeated doses of up to 3 times the maximum recommended human dose of 200 mcg/kg (on a mg/m²/day basis).

Pregnancy, Teratogenic Effects

Pregnancy Category C

Ivermectin has been shown to be teratogenic in mice, rats, and rabbits when given in repeated doses of 0.2, 8.1, and 4.5 times the maximum recommended human dose, respectively (on a mg/m²/day basis). Teratogenicity was characterized in the three species tested by cleft palate; clubbed forepaws were additionally observed in rabbits. These developmental effects were found only at or near doses that were maternotoxic to the pregnant female. Therefore, ivermectin does not appear to be selectively fetotoxic to the developing fetus. There are, however, no adequate and well-controlled studies in pregnant women. Ivermectin should not be used during pregnancy since safety in pregnancy has not been established.

Nursing Mothers

STROMECTOL is excreted in human milk in low concentrations. Treatment of mothers who intend to breastfeed should only be undertaken when the risk of delayed treatment to the mother outweighs the possible risk to the newborn.

Pediatric Use

Safety and effectiveness in pediatric patients weighing less than 15 kg have not been established.

Geriatric Use

Clinical studies of STROMEKTOL did not include sufficient numbers of subjects aged 65 and over to determine whether they respond differently from younger subjects. Other reported clinical experience has not identified differences in responses between the elderly and younger patients. In general, treatment of an elderly patient should be cautious, reflecting the greater frequency of decreased hepatic, renal, or cardiac function, and of concomitant disease or other drug therapy.

Strongyloidiasis in Immunocompromised Hosts

In immunocompromised (including HIV-infected) patients being treated for intestinal strongyloidiasis, repeated courses of therapy may be required. Adequate and well-controlled clinical studies have not been conducted in such patients to determine the optimal dosing regimen. Several treatments, i.e., at 2-week intervals, may be required, and cure may not be achievable. Control of extra-intestinal strongyloidiasis in these patients is difficult, and suppressive therapy, i.e., once per month, may be helpful.

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Swine Flu FAQ Slideshow

Overdosage & Contraindications

Body Weight (kg)	Single Oral Dose
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Number of 3-mg Tablets

15-24 1 tablet

25-35 2 tablets

36-50 3 tablets

51-65 4 tablets

66-79 5 tablets

≥ 80 200 mcg/kg

Body Weight (kg)	Single Oral Dose
------------------	------------------

Number of 3-mg Tablets

15-25 1 tablet

26-44 2 tablets

45-64 3 tablets

65-84 4 tablets

≥ 85 150 mcg/kg

OVERDOSE

Significant lethality was observed in mice and rats after single oral doses of 25 to 50 mg/kg and 40 to 50 mg/kg, respectively. No significant lethality was observed in dogs after single oral doses of up to 10 mg/kg. At these doses, the treatment-related signs that were observed in these animals include ataxia, bradypnea, tremors, ptosis, decreased activity, emesis, and mydriasis.

In accidental intoxication with, or significant exposure to, unknown quantities of veterinary formulations of ivermectin in humans, either by ingestion, inhalation, injection, or exposure to body surfaces, the following adverse effects have been reported most frequently: rash, edema, headache, dizziness, asthenia, nausea, vomiting, and diarrhea. Other adverse effects that have been reported include: seizure, ataxia, dyspnea, abdominal pain, paresthesia, urticaria, and contact dermatitis.

In case of accidental poisoning, supportive therapy, if indicated, should include parenteral fluids and electrolytes, respiratory support (oxygen and mechanical ventilation if necessary) and pressor agents if clinically significant hypotension is present. Induction of emesis and/or gastric lavage as soon as possible, followed by purgatives and other routine anti-poison measures, may be indicated if needed to prevent absorption of ingested material.

CONTRAINDICATIONS

STROMECTOL is contraindicated in patients who are hypersensitive to any component of this product.

Last updated on RxList

<http://www.faqs.org/patents/app/20080269136>

Patent title:

METHOD OF USE OF PEPTIDE ANTAGONISTS OF ZONULIN TO PREVENT OR DELAY THE ONSET OF DIABETES

Inventors: Alessio Fasano Tammara L. Watts

Agents: Connolly Bove Lodge Hutz, LLP;(FOR ALBA THERAPEUTICS)

Assignees: University of Maryland, Baltimore

Origin: WILMINGTON, DE US

IPC8 Class: AA61K3816FI

USPC Class: 514 12

ESSENCE OF ARTICLE

“The analysis of the N-termini of these molecules revealed the following common motif (amino acid residues 8-15 boxed in FIG. 1.): non-polar (Gly for intestine, Val for brain), variable, non-polar, variable, non-polar, polar, variable, polar (Gly). Gly in position 8, Val in position 12 and Gln in position 13, all are highly conserved in ZOT, zonulini and zonulinh (see FIG. 1), which is believed to be critical for receptor

binding function within the intestine. To verify the same, the synthetic octapeptide Gly Gly val Leu Val Gln Pro Gly (SEQ ID. NO: 15) (named FZI/0, and corresponding to amino acid residues 8-15 of human fetal zonulini) was chemically synthesized. “

ARTICLE

Abstract:

A method for preventing or delaying the onset of autoimmune diseases is disclosed.

Claims:

1. A method for prevention or delay of onset of an autoimmune disease, comprising: administering a pharmaceutically effective amount of a peptide antagonist of zonulin to a subject at risk of developing the autoimmune disease, wherein the peptide antagonist binds to zonula occludens toxin receptor but does not physiologically modulate the opening of mammalian intestinal tight junctions.
2. The method of claim 1, wherein the peptide antagonist comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.
3. The method of claim 1, wherein the peptide antagonist is from 8-110 amino acids in size.
4. The method of claim 1, wherein the peptide antagonist is from 8-40 amino acids in size.
5. The method of claim 1, wherein the peptide antagonist comprises amino acid sequence SEQ ID NO: 15.
6. The method of claim 1, wherein the peptide antagonist consists of amino acid sequence SEQ ID NO: 15.
7. The method of claim 1, wherein the peptide antagonist is administered as an oral dosage composition for intestinal delivery.
8. The method of claim 1, wherein the peptide antagonist consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

9. A method for prevention or delay of onset of an autoimmune disease, comprising: administering a pharmaceutically effective amount of an antagonist of zonulin to a subject at risk of developing the autoimmune disease, wherein the antagonist binds to zonula occludens toxin receptor but does not physiologically modulate the opening of mammalian intestinal tight junctions.

10. The method of claim 9, wherein the antagonist comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

11. The method of claim 9, wherein the antagonist comprises a peptide comprising 8-110 amino acids.

12. The method of claim 9, wherein the antagonist comprises a peptide comprising 8-40 amino acids.

13. The method of claim 9, wherein the antagonist comprises amino acid sequence SEQ ID NO: 15.

14. The method of claim 9, wherein the antagonist consists of amino acid sequence SEQ ID NO: 15.

15. The method of claim 9, wherein the antagonist is administered as an oral dosage composition for intestinal delivery.

16. The method of claim 9, wherein the antagonist comprises a peptide, wherein the peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

17. A method for prevention of diabetes, comprising: administering to a subject in need of such prevention, a pharmaceutically effective amount of a peptide antagonist of zonulin, wherein the peptide antagonist binds to zonula occludens toxin receptor but does not physiologically modulate the opening of mammalian intestinal tight junctions.

18. The method of claim 17, wherein the peptide antagonist comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

19. The method of claim 17, wherein the peptide antagonist is from 8-110 amino acids in size.

20. The method of claim 17, wherein the peptide antagonist is from 8-40 amino acids in size.

21. The method of claim 17, wherein the peptide antagonist comprises amino acid sequence SEQ ID NO: 15.
22. The method of claim 17, wherein the peptide antagonist consists of amino acid sequence SEQ ID NO: 15.
23. The method of claim 17, wherein the peptide antagonist is administered as an oral dosage composition for intestinal delivery.
24. The method of claim 17, wherein the peptide antagonist consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.
25. A method for prevention or delay of onset of diabetes, comprising: administering a pharmaceutically effective amount of an antagonist of zonulin to a subject at risk of developing diabetes, wherein the antagonist binds to zonula occludens toxin receptor but does not physiologically modulate the opening of mammalian intestinal tight junctions.
26. The method of claim 25, wherein the antagonist comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.
27. The method of claim 25, wherein the antagonist comprises a peptide comprising 8-110 amino acids.
28. The method of claim 25, wherein the antagonist comprises a peptide comprising 8-40 amino acids.
29. The method of claim 25, wherein the antagonist comprises amino acid sequence SEQ ID NO: 15.
30. The method of claim 25, wherein the antagonist consists of amino acid sequence SEQ ID NO: 15.
31. The method of claim 25, wherein the antagonist is administered as an oral dosage composition for intestinal delivery.
32. The method of claim 25, wherein the antagonist comprises a peptide, wherein the peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

Description:

FIELD OF THE INVENTION

[0002]The present invention relates to use of peptide antagonists of zonulin to prevent or delay the onset of diabetes, particularly type I diabetes. The peptide antagonists bind to the zonula occludens toxin receptor, yet do not physiologically modulate the opening of mammalian tight junctions.

BACKGROUND OF THE INVENTION

[0003]I. Function and Regulation of Intestinal Tight Junctions

[0004]The intestinal epithelium represents the largest interface (more than 2,000,000 cm²) between the external environment and the internal milieu. The maintenance of intercellular tight junctions ("tj") competence prevents movements of potentially harmful environmental factors, such as bacteria, viruses, toxins, food allergens, and macromolecules across the intestinal barrier. This competence is significantly jeopardized in a variety of clinical conditions affecting the gastrointestinal tract, including food allergies, enteric infections, malabsorption syndromes, and inflammatory bowel diseases. The tj or zonula occludens (hereinafter "ZO") are one of the hallmarks of absorptive and secretory epithelia (Madara, *J. Clin. Invest.*, 83:1089-1094 (1989); and Madara, *Textbook of Secretory Diarrhea* Eds. Leberthal et al, Chapter 11, pages 125-138 (1990). As a barrier between apical and basolateral compartments, they selectively regulate the passive diffusion of ions and water-soluble solutes through the paracellular pathway (Gumbiner, *Am. J. Physiol.*, 253 (Cell Physiol. 22): C749-C758 (1987)). This barrier maintains any gradient generated by the activity of pathways associated with the transcellular route (Diamond, *Physiologist*, 20:10-18 (1977)).

[0005]Variations in transepithelial conductance can usually be attributed to changes in the permeability of the paracellular pathway, since the resistances of enterocyte plasma membranes are relatively high (Madara (1989, 1990), supra). The ZO represents the major barrier in this paracellular pathway, and the electrical resistance of epithelial tissues seems to depend on the number of transmembrane protein strands, and their complexity in the ZO, as observed by freeze-fracture electron microscopy (Madara et al, *J. Cell Biol.*, 101:2124-2133 (1985)).

[0006]There is abundant evidence that ZO, once regarded as static structures, are in fact dynamic and readily adapt to a variety of developmental (Magnuson et al, *Dev. Biol.*, 67:214-224 (1978); Revel et al, *Cold Spring Harbor Symp. Quant. Biol.*, 40:443-455 (1976); and Schneeberger et al, *J. Cell Sci.*, 32:307-324 (1978)), physiological (Gilula et al, *Dev. Biol.*, 50:142-168 (1976); Madara et al, *J. Membr. Biol.*, 100:149-164 (1987); Mazariegos et al, *J. Cell Biol.*, 98:1865-1877 (1984); and Sardet et al, *J. Cell Biol.*, 80:96-117 (1979)), and pathological (Milks et al, *J. Cell Bio.*, 103:2729-2738 (1986); Nash et al, *Lab. Invest.*, 59:531-537 (1988); and Shasby et al, *Am. J. Physiol.*, 255 (Cell Physiol., 24:C781-C788 (1988)) circumstances. The regulatory mechanisms that Underlie this adaptation are still not completely understood. However, it is clear that, in the presence of Ca²⁺, assembly of the ZO is the result of cellular interactions that trigger a complex cascade of biochemical events that ultimately lead to the formation and modulation of an organized network of ZO elements, the composition of which has been only partially characterized (Diamond, *Physiologist*, 20:10-18 (1977)). A candidate for the transmembrane protein strands, occludin, has recently been identified (Puruse et al, *J. Membr. Biol.*, 87:141-150 (1985)).

[0007]Six proteins have been identified in a cytoplasmic submembranous plaque underlying membrane contacts, but their function remains to be established (Diamond, supra). ZO-1 and ZO-2 exist as a heterodimer (Gumbiner et al, Proc. Natl. Acad. Sci., USA, 88:3460-3464 (1991)) in a detergent-stable complex with an uncharacterized 130 kD protein (ZO-3). Most immunoelectron microscopic studies have localized ZO-1 to precisely beneath membrane contacts (Stevenson et al, Molec. Cell Biochem., 83:129-145 (1988)). Two other proteins, cingulin (Citi et al, Nature (London), 333:272-275 (1988)) and the 7H6 antigen (Zhong et al, J. Cell Biol., 120:477-483 (1993)) are localized further from the membrane and have not yet been cloned. Rab 13, a small GTP binding protein has also recently been localized to the junction region (Zahraoui et al, J. Cell Biol., 124:101-115 (1994)). Other small GTP-binding proteins are known to regulate the cortical cytoskeleton, i.e., rho regulates actin-membrane attachment in focal contacts (Ridley et al, Cell, 70:389-399 (1992)), and rac regulates growth factor-induced membrane ruffling (Ridley et al, Cell, 70:401-410 (1992)). Based on the analogy with the known functions of plaque proteins in the better characterized cell junctions, focal contacts (Guan et al, Nature, 358:690-692 (1992)), and adherens junctions (Tsukita et al, J. Cell Biol., 123:1049-1053 (1993)), it has been hypothesized that tj-associated plaque proteins are involved in transducing signals in both directions across the cell membrane, and in regulating links to the cortical actin cytoskeleton.

[0008]To meet the many diverse physiological and pathological challenges to which epithelia are subjected, the ZO must be capable of rapid and coordinated responses that require the presence of a complex regulatory system. The precise characterization of the mechanisms involved in the assembly and regulation of the ZO is an area of current active investigation.

[0009]There is now a body of evidence that tj structural and functional linkages exist between the actin cytoskeleton and the tj complex of absorptive cells (Gumbiner et al, supra; Madara et al, supra; and Drenchahn et al, J. Cell Biol., 107:1037-1048 (1988)). The actin cytoskeleton is composed of a complicated meshwork of microfilaments whose precise geometry is regulated by a large cadre of actin-binding proteins. An example of how the state of phosphorylation of an actin-binding protein might regulate cytoskeletal linking to the cell plasma membrane is the myristoylated alanine-rich C kinase substrate (hereinafter "MARCKS"). MARCKS is a specific protein kinase C (hereinafter "PKC") substrate that is associated with the cytoplasmic face of the plasma membrane (Aderem, Elsevier Sci. Pub. (UK), pages 438-443 (1992)). In its non-phosphorylated form, MARCKS crosslinks to the membrane actin. Thus, it is likely that the actin meshwork associated with the membrane via MARCKS is relatively rigid (Hartwig et al, Nature, 356:618-622 (1992)). Activated PKC phosphorylates MARCKS, which is released from the membrane (Rosen et al, J. Exp. Med., 172:1211-1215 (1990); and Thelen et al, Nature, 351:320-322 (1991)). The actin linked to MARCKS is likely to be spatially separated from the membrane and be more plastic. When MARCKS is dephosphorylated, it returns to the membrane where it once again crosslinks actin (Hartwig et al, supra; and Thelen et al, supra). These data suggest that the F-actin network may be rearranged by a PKC-dependent phosphorylation process that involves actin-binding proteins (MARCKS being one of them).

[0010]A variety of intracellular mediators have been shown to alter tj function and/or structure. Tight junctions of amphibian gallbladder (Duffey et al, Nature, 204:451-452 (1981)), and both goldfish (Bakker et al, Am. J. Physiol., 246G213-G217 (1984)) and flounder (Krasney et al, Fed. Proc., 42:1100 (1983)) intestine, display enhanced resistance to passive ion flow as intracellular cAMP is elevated. Also, exposure of amphibian gallbladder to Ca²⁺ ionophore appears to enhance tj resistance, and induce

alterations in *tj* structure (Palant et al, *Am. J. Physiol.*, 245:C203-C212 (1983)). Further, activation of PKC by phorbol esters increases paracellular permeability both in kidney (Ellis et al, *C. Am. J. Physiol.*, 263 (Renal Fluid Electrolyte Physiol. 32):F293-F300 (1992)), and intestinal (Stenson et al, *C. Am. J. Physiol.*, 265 (Gastrointest. Liver Physiol., 28):G955-G962 (1993)) epithelial cell lines. [0011]II. Zonula Occludens Toxin

[0012]Most *Vibrio cholerae* vaccine candidates constructed by deleting the *ctxA* gene encoding cholera toxin (CT) are able to elicit high antibody responses, but more than one-half of the vaccinees still develop mild diarrhea (Levine et al, *Infect. Immun.*, 56(1):161-167 (1988)). Given the magnitude of the diarrhea induced in the absence of CT, it was hypothesized that *V. cholerae* produce other enterotoxigenic factors, which are still present in strains deleted of the *ctxA* sequence (Levine et al, *supra*). As a result, a second toxin, zonula occludens toxin (hereinafter "ZOT") elaborated by *V. cholerae* and which contribute to the residual diarrhea, was discovered (Fasano et al, *Proc. Natl. Acad. Sci., USA*, 8:5242-5246 (1991)). The *zot* gene is located immediately adjacent to the *ctx* genes. The high percent concurrence of the *zot* gene with the *ctx* genes among *V. cholerae* strains (Johnson et al, *J. Clin. Microb.*, 31/3:732-733 (1993); and Karasawa et al, *FEBS Microbiology Letters*, 106:143-146 (1993)) suggests a possible synergistic role of ZOT in the causation of acute dehydrating diarrhea typical of cholera. Recently, the *zot* gene has also been identified in other enteric pathogens (Tschape; 2nd Asian-Pacific Symposium on Typhoid fever and other Salmonellosis, 47(Abstr.) (1994)).

[0013]It has been previously found that, when tested on rabbit ileal mucosa, ZOT increases the intestinal permeability by modulating the structure of intercellular *tj* (Fasano et al, *supra*). It has been found that as a consequence of modification of the paracellular pathway, the intestinal mucosa becomes more permeable. It also was found that ZOT does not affect Na⁺-glucose coupled active transport, is not cytotoxic, and fails to completely abolish the transepithelial resistance (Fasano et al, *supra*).

[0014]More recently, it has been found that ZOT is capable of reversibly opening *tj* in the intestinal mucosa, and thus ZOT, when co-administered with a therapeutic agent, e.g., insulin, is able to effect intestinal delivery of the therapeutic agent, when employed in an oral dosage composition for intestinal drug delivery, e.g., in the treatment of diabetes (WO 96/371.6; U.S. Pat. No. 5,827,534; U.S. Pat. No. 5,665,389; and Fasano et al, *J. Clin. Invest.*, 99:1158-1164 (1997); each of which is incorporated by reference herein in their entirety). It has also been found that ZOT is capable of reversibly opening *tj* in the nasal mucosa, and thus ZOT, when co-administered with a therapeutic agent, is able to enhance nasal absorption of a therapeutic agent (U.S. Pat. No. 5,908,825; which is incorporated by reference herein in its entirety).

[0015]In U.S. Pat. No. 5,864,014; which is incorporated by reference herein in its entirety, a ZOT receptor has been identified and purified from an intestinal cell line, i.e., CaCo2 cells. Further, in U.S. Pat. No. 5,912,323; which is incorporated by reference herein in its entirety, ZOT receptors from human intestinal, heart and brain tissue have been identified and purified. The ZOT receptors represent the first step of the paracellular pathway involved in the regulation of intestinal and nasal permeability. [0016]III. Zonulin

[0017]In U.S. Pat. Nos. 5,545,510 and 5,948,629, which are incorporated by reference herein in their entirety, mammalian proteins that are immunologically and functionally related to ZOT, and that function as the physiological modulator of mammalian tight junctions, have been identified and purified. These mammalian proteins, referred to as "zonulin", are useful for enhancing absorption of therapeutic agents across tj of intestinal and nasal mucosa, as well as across tj of the blood brain barrier. [0018]IV. Peptide Antagonists of Zonulin

[0019]Peptide antagonists of zonulin were identified and described for the first time in pending U.S. patent application Ser. No. 09/127,815, filed Aug. 3, 1998, which is incorporated by reference herein in its entirety, which corresponds to WO 00/07609. Said peptide antagonists bind to the ZOT receptor, yet do not function to physiologically modulate the opening of mammalian tight junctions. The peptide antagonists competitively inhibit the binding of ZOT and zonulin to the ZOT receptor, thereby inhibiting the ability of ZOT and zonulin to physiologically modulate the opening of mammalian tight junctions.

[0020]V. Diabetes

[0021]The morbidity and mortality associated with diabetes is devastating. The total number of diabetic individuals in the United States is 15.7 million. Of these, 100% of the type I diabetic individuals and 40% of type II diabetic individuals depend on paternal administration of insulin. On an annual basis, the direct medical costs associated with diabetes exceeds 40 billion dollars. An additional 14 billion dollars is associated with disability, work loss, and premature mortality.

[0022]Although oral insulin drug delivery strategies have been the focus of many research efforts, they have been largely unsuccessful because the physiologic nature of the small intestine prevents the absorption of macromolecules, such as insulin.

[0023]An oral dosage composition comprising ZOT for targeting delivery of insulin to the paracellular pathway for the treatment of diabetes has been described in U.S. Pat. Nos. 5,827,534 and 5,665,389. By physiologically modulating the paracellular pathway using ZOT, it is now possible to introduce a wide variety of therapeutic agents into the systemic circulation. This drug delivery system adds targeting specificity, which has long hampered the design of many oral pharmaceutical agents. The utility of this system is not limited to insulin delivery, and may represent a new way of designing orally administered pharmaceutical agents.

[0024]While offering an innovative treatment strategy for a disease as debilitating as diabetes is promising, preventing or delaying the onset of disease has widespread implications. Understanding the pathogenesis of any disease process is a daunting task. Heretofore, there has been no prior evidence of a pharmaceutical agent with the capability of preventing or delaying the onset of diabetes. In the present invention new light has been shed on the pathogenesis, prevention and delaying of onset of diabetes by demonstrating that a critical and early step in disease progression resides in alterations in paracellular permeability. In the present invention, it has been demonstrated that an increase in paracellular permeability is necessary for the progression toward diabetes. Peptide antagonists of zonulin, which block this endogenous pathway, have been found in the present invention to prevent the progression to diabetes. Thus, the present invention is believed to be useful to prevent long-term complications of diabetes. Further, the permeability changes associated with autoimmune diseases are long standing, and early intervention per the present invention is believed to have untold benefits to the diabetic patient.

SUMMARY OF THE INVENTION

[0025]An object of the present invention is to provide a method for the prevention or delay the onset of diabetes.

[0026]This and other objects of the present invention, which will be apparent from the detailed description of the invention provided hereinafter, have been met, in one embodiment, by a method for preventing or delay the onset of diabetes (particularly, type I diabetes) comprising administering to a subject in need of such prevention or delay of onset, a pharmaceutically effective amount of a peptide antagonist of zonulin, wherein said peptide antagonist binds to ZOT receptor, yet does not physiologically modulate the opening of mammalian tight junctions.

BRIEF DESCRIPTION. OF THE DRAWINGS

[0027]FIG. 1 shows a comparison of the N-terminal sequences of zonulin purified from various human tissues and IgM heavy chain with the N-terminal sequence of the biologically active fragment (amino acids 288-399) of ZOT.

[0028]FIG. 2 shows the effect of ZoT, zonulini, zonulinh, either alone (closed bars), or in combination with the peptide antagonist FZI/0 (open bars) or in combination with FZI/1 (shaded bars), as compared to the negative control, on the tissue resistance (Rt) of rabbit ileum mounted in Ussing chambers. N equals 3-5; and * equals $p < 0.01$.

[0029]FIG. 3 shows the concentrations (ng/ml) of intraluminal zonulin in both diabetic-prone and diabetic-resistant rats, which was determined using a sandwich ELISA assay. Samples were obtained by intestinal lavage in normal saline. The first bar in each case represents diabetic-resistant rats (DR). The second bar represents diabetic-prone animals (DP), and the third bar represents rats with chronic diabetes (CD). $< 9\%$ of the diabetic-prone rats do not become diabetic, and $< 9\%$ of the diabetic-resistant rats develop diabetes.

[0030]FIG. 4 shows the percentage of rats used in the study that progressed to diabetes.

[0031]FIG. 5 shows the concentrations (ng/ml) of intraluminal zonulin in diabetic rats, which was determined using a sandwich ELISA assay.

[0032]FIG. 6 shows ex vivo intestinal permeability in diabetic resistant (DR) rats, untreated diabetic-prone rats (DP-untreated; second bar) determined in Ussing chambers, diabetic-prone rats treated with the peptide antagonist of zonulin (DP-treated; third bar). * equals $p < 0.05$; ** equals $p < 0.05$, and $p < 0.001$ compared to DP-treated.

[0033]FIG. 7 shows ex vivo intestinal permeability in the small intestines of untreated, diabetes-prone rats that either developed or did not develop diabetes. * equals $p < 0.04$.

DETAILED DESCRIPTION OF THE INVENTION

[0034]As discussed above, in one embodiment, the above-described object of the present invention have been met by a method for preventing or delaying the onset of diabetes (particularly, type I diabetes) comprising administering to a subject in need of such prevention or delay of onset, a pharmaceutically effective amount of a peptide antagonist of zonulin, wherein said peptide antagonist binds to ZOT receptor, yet does not physiologically modulate the opening of mammalian tight junctions

[0035]The particular peptide antagonist of zonulin employed in the present invention is not critical thereto. Examples of said peptide antagonists include peptides which comprise an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, and SEQ ID NO: 24.

[0036]The size of the peptide antagonist is not critical to the present invention. Generally, the size of the peptide antagonist will range from 8 to 110, amino acids, preferably from 8 to 40 amino acids, more preferably will be 8 amino acids.

[0037]The peptide antagonists can be chemically synthesized and purified using well-known techniques, such as described in High Performance Liquid Chromatography of Peptides and Proteins: Separation Analysis and Conformation, Eds. Mant et al, C.R.C. Press (1991), and a peptide synthesizer, such as Symphony (Protein Technologies, Inc); or by using recombinant DNA techniques, i.e., where the nucleotide sequence encoding the peptide is inserted in an appropriate expression vector, e.g., an E. coli or yeast expression vector, expressed in the respective host cell, and purified therefrom using well-known techniques.

[0038]The peptide antagonists can be administered as oral dosage compositions for small intestinal delivery. Such oral dosage compositions for small intestinal delivery are well-known in the art, and generally comprise gastroresistant tablets or capsules (Remington's Pharmaceutical Sciences, 16th Ed., Eds. Osol, Mack Publishing Co., Chapter 89 (1980); Digenis et al, J. Pharm. Sci., 83:915-921 (1994); Vantini et al, Clinica Terapeutica, 145:445-451 (1993); Yoshitomi et al, Chem. Pharm. Bull., 40:1902-1905 (1992); Thoma et al, Pharmazie, 46:331-336 (1991); Morishita et al, Drug Design and Delivery, 7:309-319 (1991); and Lin et al, Pharmaceutical Res., 8:919-924 (1991)); each of which is incorporated by reference herein in its entirety).

[0039]Tablets are made gastroresistant by the addition of, e.g., either cellulose acetate phthalate or cellulose acetate terephthalate.

[0040]Capsules are solid dosage forms in which the peptide antagonist(s) is enclosed in either a hard or soft, soluble container or shell of gelatin. The gelatin used in the manufacture of capsules is obtained from collagenous material by hydrolysis. There are two types of gelatin. Type A, derived from pork skins by acid processing, and Type B, obtained from bones and animal skins by alkaline processing. The use of hard gelatin capsules permit a choice in prescribing a single peptide antagonist or a combination thereof at the exact dosage level considered best for the individual subject. The hard gelatin capsule consists of two sections, one slipping over the other, thus completely surrounding the peptide antagonist. These capsules are filled by introducing the peptide antagonist, or gastroresistant beads containing the peptide antagonist,

into the longer end of the capsule, and then slipping on the cap. Hard gelatin capsules are made largely from gelatin, FD&C colorants, and sometimes an opacifying agent, such as titanium dioxide. The USP permits the gelatin for this purpose to contain 0.15% (w/v) sulfur dioxide to prevent decomposition during manufacture.

[0041]In the context of the present invention, oral dosage compositions for small intestinal delivery also include liquid compositions which contain aqueous buffering agents that prevent the peptide antagonist from being significantly inactivated by gastric fluids in the stomach, thereby allowing the peptide antagonist to reach the small intestines in an active form. Examples of such aqueous buffering agents which can be employed in the present invention include bicarbonate buffer (pH 5.5 to 8.7, preferably about pH 7.4).

[0042]When the oral dosage composition is a liquid composition, it is preferable that the composition be prepared just prior to administration so as to minimize stability problems. In this case, the liquid composition can be prepared by dissolving lyophilized peptide antagonist in the aqueous buffering agent.

[0043]The pharmaceutically effective amount of peptide antagonist employed is not critical to the present invention and will vary depending upon the age, weight and sex of the subject being treated. Generally, the amount of peptide antagonist employed in the present invention to prevent or delay the onset of diabetes, is in the range of about 7.5×10^{-6} M to 7.5×10^{-3} M, preferably about 7.5×10^{-6} M to 7.5×10^{-4} M. To achieve such a final concentration in, e.g., the intestines or blood, the amount of peptide antagonist in a single oral dosage composition of the present invention will generally be about 1.0 μ g to 1000 μ g, preferably about 1.0 μ g to 100 μ g.

[0044]The following examples are provided for illustrative purposes only, and are in no way intended to limit the scope of the present invention.

EXAMPLE 1

Peptide Antagonists of Zonulin

[0045]Given that ZOT, human intestinal zonulin (zonulini) and human heart zonulin (zonulinh) all act on intestinal (Fasano et al, Gastroenterology, 112:839 (1997); Fasano et al, J. Clin. Invest., 96:710 (1995)) and endothelial tj and that all three have a similar regional effect (Fasano et al (1997), supra) that coincides with the ZOT receptor distribution within the intestine (Fasano et al (1997), supra; and Fasano et al (1995), supra), it was postulated in U.S. patent application Ser. No. 09/127,815, filed Aug. 3, 1998, that these three molecules interact with the same receptor binding site. A comparison of the primary amino acid structure of ZOT and the human zonulins was thus carried out therein to provide insights as to the absolute structural requirements of the receptor-ligand interaction involved in the regulation of intestinal tj. The analysis of the N-termini of these molecules revealed the following common motif (amino acid residues 8-15 boxed in FIG. 1.): non-polar (Gly for intestine, Val for brain), variable, non-polar, variable, non-polar, polar, variable, polar (Gly). Gly in position 8, Val in position 12 and Gln in position 13, all are highly conserved in ZOT, zonulini and zonulinh (see FIG. 1), which is believed to be critical for receptor binding function within the intestine. To verify the same, the synthetic octapeptide

Gly Gly val Leu Val Gln Pro Gly (SEQ ID. NO: 15) (named FZI/0, and corresponding to amino acid residues 8-15 of human fetal zonulini) was chemically synthesized.

[0046]Next, rabbit ileum -mounted in Ussing chambers as described above, were exposed to 100 µg of FZI/0 (SEQ ID NO: 15), 100 µg of FZI/1 (SEQ ID NO: 29), 1.0 µg of 6xHis-ZOT (obtained as described in Example 1 of U.S. patent application Ser. No. 09/127,815, filed Aug. 3, 1998), 1.0 µg of sonulini (obtained as described in Example 3 of U.S. patent application Ser. No. 09/127,815, filed Aug. 3, 1998), or 1.0 µg of zonulinh (obtained as described in Example 3 of U.S. patent application Ser. No. 09/127,815, filed Aug. 3, 1998), alone; or pre-exposed for 20 min to 100 µg of FZI/0 or FZI/1, at which time 1.0 µg of 6xHis-ZOT, 1.0 µg of zonulini, or 1.0 µg of zonulinh, was added. ΔRt was then calculated as described above. The results are shown in FIG. 2.

[0047]As shown in FIG. 2, FZI/0 did not induce any significant change in Rt (0.5% as compared to the negative control) (see closed bar). On the contrary, pre-treatment for 20 min with FZI/0 decreased the effect of ZOT, zonulini, and zonulinh on Rt by 75%, 97%, and 100%, respectively (see open bar). Also as shown in FIG. 2, this inhibitory effect was completely ablated when a second synthetic peptide (FZI/1, SEQ ID NO: 29) was chemically synthesized by changing the Gly in position 8, the Val in position 12, and the Gln in position 13 (as referred to zonulini) with the correspondent amino acid residues of zonulinb (Val, Gly, and Arg, respectively, see SEQ ID NO: 30) was used (see shaded bar).

[0048]The above results demonstrate that there is a region spanning between residue 8 and 15 of the N-terminal end of ZOT and the zonulin family that is crucial for the binding to the target receptor, and that the amino acid residues in position 8, 12 and 13 determine the tissue specificity of this binding.

EXAMPLE 2

Diabetic Rat Model

[0049]Alterations in intestinal permeability have been shown to be one of the preceding physiologic changes associated with the onset of diabetes (Meddings, *Am. J. Physiol.*, 276:G951-957 (1999)). Paracellular transport and intestinal permeability is regulated by intracellular tj via mechanisms which have not been completely elucidated.

[0050]Zonulin and its prokaryotic analog, ZOT, both alter intestinal permeability by modulating tj. In this example, it has been demonstrated for the first time that zonulin-related impairment of tj is involved in the pathogenesis of diabetes, and that diabetes can be prevented, or the onset delayed, by administration of a peptide antagonist of zonulin.

[0051]Initially, two genetic breeds, i.e., BB/Wor diabetic-prone (DP) and diabetic-resistant (DR) rats (Haber et al, *J. Clin. Invest.*, 95:832-837 (1993)), were evaluated to determine whether they exhibited significant changes in intraluminal secretion of zonulin and intestinal permeability.

[0052]More specifically, age-matched DP and DR rats (20, 50, 75, and >100 days of age) were sacrificed. After the rats were sacrificed, a 25 G needle was placed within the lumen of the ileum, and intestinal lavage with Ringer's solution was performed to determine the presence of intraluminal zonulin. Zonulin concentration was evaluated using a sandwich enzyme linked immunosorbent assay (ELISA) as follows:

[0053]Plastic microtiter plates (Costar, Cambridge, Mass.) were coated with polyclonal rabbit anti-ZOT antibodies (obtained as described in Example 2 of U.S. application Ser. No. 09/127,815 filed Aug. 3, 1998) (dilution 1:100) overnight at 4° C., washed three times with PBS containing 0.05% (v/v) Tween 20, then blocked by incubation with 300 µl of PBS containing 0.1% (v/v) Tween 20, for 15 min at room temperature. Next, purified human intestine zonulin (obtained as described in Example 3 of U.S. application Ser. No. 09/127,815 filed Aug. 3, 1998) was coated on the plates.

[0054]A standard curve was obtained by diluting zonulin in PBS containing 0.05% (v/v) Tween 20 at different concentration: 0.78 ng/ml, 1.56 ng/ml, 3.125 ng/ml, 6.25 ng/ml, 12.5 ng/ml, 25 ng/ml and 50 ng/ml.

[0055]100 µl of each standard concentration or 100 µl of intestinal lavage sample were pipetted into the wells, and incubated for 1 hr at room temperature, using a plate shaker. Unbound zonulin was washed-out using PBS, and the wells were incubated with 100 µl of anti-ZOT antibodies conjugated with alkaline phosphate for 1 hr at room temperature with shaking. Unbound conjugate was washed-out with PBS, and a color reaction was developed by first adding 100 µl of Extra-Avidin (SIGMA, St. Louis, Mo.) diluted 1/20000 in 0.1 M Tris-HCl (pH 7.3), 1.0 mM MgCl₂, 1.0% (w/v) BSA for 15 min, and then incubating each well for 30 min at 37° C. with 100 µl of a solution containing 1.0 mg/ml of p-nitrophenyl-phosphate substrate (SIGMA, St. Louis, Mo.). Absorbance was read on an enzyme immunoassay reader at 405 nm.

[0056]In order to evaluate the intra- and inter-assay precision of the ELISA-sandwich method, the coefficient variation (CV) was calculated using three replicates from two samples with different concentrations of zonulin, on three consecutive days. The inter-assay test of the ELISA-sandwich method produced CV values of 9.8%, The CV of the intra-assay test was 4.2% at day 1, 3.3% at day 2 and 2.9% at day 3.

[0057]Zonulin concentration was expressed as ng/mg protein detected in the intestinal lavages and normalized by exposed surface area (in mm²). The results are shown in FIG. 3.

[0058]As shown in FIG. 3, a 4-fold increase in intraluminal zonulin was first observed in diabetic-prone, rats (age 50 days) (second bar). This increase in intraluminal zonulin was found to correlate with an increase in intestinal permeability. The increase in intraluminal zonulin remains high in these diabetic-prone rats, and found to correlate with the progression toward full-blown diabetes. Of note, the diabetic-prone rat (age >100 days) did not have an increase in intraluminal zonulin. This is remarkable, as this rat did not progress to diabetes. Blood glucose for this rat was normal. Thus, zonulin is responsible for the permeability changes associated with the pathogenesis of type I diabetes. The increase in zonulin secretion is age-related, and proceeds the onset of diabetes.

[0059]Next, in order to demonstrate that diabetes can be prevented by administration of a peptide antagonist of zonulin, BB/Wor rats (ages 21-26 days), were obtained from Biomedical Research Models, Inc. (Rutland, Mass.), and were randomized into two groups (n=5 per group), i.e., a treated group and a control group. Both groups were maintained on a standard diet of rat chow (Harlan Teklab Diet #7012). All food and water were previously autoclaved. Each day, daily water intake was measured and 100 ml of

fresh water was given. The treated group received 10 µg/ml of the zonulin peptide antagonist (SEQ ID NO: 15) supplemented in the drinking water. The rats were housed in hepa-filter cages.

[0060]Diabetes in the rats was diagnosed as follows: The rats were weighed twice a week. Blood glucose was determined weekly using the OneTouch® glucose monitoring system (Johnson & Johnson). Each week, reagent strips for urinalysis were used to monitor glucose (Diastix®) and ketones (Ketositx®) (Bayer). Rats with a blood glucose >250 mg/dl were fasted overnight, and blood glucose levels >200 mg/dl were considered diabetic. These guidelines are in accordance with the data supplied by Biomedical Research Models, Inc. The results are shown in FIG. 4.

[0061]As shown in FIG. 4, 80% of the control rats (4/5) and 40% of the rats treated with the peptide antagonist of zonulin (2/5) developed diabetes by age 80 days. Alterations in zonulin secretion paralleled the onset of diabetes.

[0062]Following clinical presentation of diabetes, the rats were sacrificed as follows: the rats were anesthetized using ketamine anesthesia and a midline incision was made allowing access to the heart. An 18G needle was placed into the heart and death occurred by exsanguinations. Then, zonulin assays were conducted as described above. For those rats that did not present with diabetes, the endpoint of the study was age 80 days. According to Biomedical Research Models, Inc., 80% of diabetes prone rats present with diabetes by age 80 days. The results of the zonulin assays are shown in FIG. 5.

[0063]As shown in FIG. 5, the diabetic rats that were not treated with the peptide antagonist of zonulin were observed to have an increase in intraluminal zonulin, which was consistent with the results shown in FIG. 3. Further, intraluminal zonulin was increased 2 to 4-fold in diabetic rats (DR), as compared to both diabetic-prone rats that did not develop diabetes (DP-treated) and control rats (DP-untreated). Non-diabetic control rats that did not develop diabetes had negligible levels of zonulin, consistent with the levels of zonulin shown in FIG. 3. Moreover, two diabetic-prone rats that developed diabetes despite treatment with the peptide antagonist of zonulin showed intraluminal zonulin levels that were significantly higher than the successfully treated rats, and the untreated control rats. The levels of zonulin were sufficient to initiate the permeability changes necessary to progress to diabetes, but the ZOT/zonulin receptors were effectively blocked by the peptide antagonist.

[0064]Also, following clinical presentation of diabetes, the intestinal tissues of the sacrificed rats were mounted in Ussing chamber to assess for changes in ex vivo permeability.

[0065]More specifically, sections of jejunum and ileum were isolated from the sacrificed rats, and rinsed free of intestinal contents. Six sections of each intestinal segment was prepared and mounted in Lucite Ussing chambers (0.33 cm³ opening), connected to a voltage clamp apparatus (EVC 4000; World Precision Instruments, Sarasota, Fla.), and bathed with freshly prepared buffer comprising 53 mM NaCl, 5.0 mM KCl, 30.5 mM Na₂SO₄, 30.5 mM mannitol, 1.69 mM Na₂PO₄, 0.3 mM NaHPO₄, 1.25 mM CaCl₂, 1.1 mM MgCl₂, and 25 mN NaHCO₃ (pH 7.4). The bathing solution was maintained at 37° C. with water-jacketed reservoirs connected to a constant temperature circulating pump and gassed with 95% O₂ and 5% CO₂. Potential difference was measured and short-circuit current and tissue resistance was calculated as described by Fasano et al, Proc. Natl. Acad. Sci.USA, 88:5242-5246 (1991). The results are shown in FIGS. 6-7.

[0066]As demonstrated in the ex vivo Ussing chamber permeability studies, and shown in FIG. 6, all of the rats that progressed to diabetes had an increase in their intestinal permeability. Diabetic resistant (DR) rats had no appreciable alterations in paracellular permeability (first bar). Untreated diabetic-prone rats (DP-untreated; second bar) had a significant increase in paracellular permeability of the jejunum and ileum. More importantly, diabetic-prone rats treated with the peptide antagonist of zonulin (DP-treated; third bar) had a significant increase in paracellular permeability of the small intestine restricted to the jejunum. However, as shown in FIG. 6, pre-treatment with the zonulin peptide antagonist prevented these changes in the distal ileum. Consequently, alterations in paracellular permeability associated with the pathogenesis are restricted to the ileum. Also, as shown in FIG. 6, there are no significant changes in permeability of the colon, which coincides, with the regional distribution of the zonulin receptor distribution.

[0067]These results were further validated by a comparison of ex vivo intestinal permeability in the small intestines of untreated diabetes-prone rats that either developed (DP-D) or did not developed (DP-N) diabetes (FIG. 7). While no significant changes in jejunal Rt were observed between DP-D and DP-N rats, a significant lower Rt of the ileal mucosa of DP-D rats was observed as compared to DP-N rats (FIG. 7).

[0068]Thus, the following conclusions can be made: (1) the peptide antagonist was able to effectively block the permeability changes required for the development of diabetes; and (2) in those rats treated with the peptide antagonist, the levels of Intraluminal zonulin are 3-fold higher than the treated rats that did not develop diabetes. In this population of treated rats that developed diabetes, the amount of peptide antagonist may not have been enough to block a sufficient number of ZOT/zonulin receptors necessary to prevent diabetes.

[0069]60% of the treated rats did not develop diabetes. In this population of rats, the peptide antagonist of zonulin effectively prevented the increase in intestinal permeability necessary for the onset of diabetes. As shown in FIG. 5, the treated rats had levels of intraluminal zonulin comparable with the untreated controls, but due to the presence of the peptide antagonist of zonulin, the overall permeability the small intestine was not altered enough to initiate the pathophysiologic changes necessary for the progression to diabetes. Interestingly, as shown in FIG. 5, the one control animal that did not develop diabetes had negligible levels of zonulin, further supporting the role of zonulin in the pathogenesis of diabetes.

[0070]Accordingly, an early event in the pathogenesis of diabetes in BB/Wor rats involves changes in zonulin-mediated Intestinal paracellular permeability. Furthermore, inhibition of the zonulin signaling system with the use of peptide antagonists of zonulin prevents, or at least delays, the onset of diabetes.

[0071]While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one of ordinary skill in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Sequence CWU 1

3318PRTArtificialtight junction antagonist peptide 1Gly Arg Val Cys Val Gln Pro Gly1

528PRTArtificialtight junction antagonist peptide 2Gly Arg Val Cys Val Gln Asp Gly1

538PRTArtificialtight junction antagonist peptide 3Gly Arg Val Leu Val Gln Pro Gly1

548PRTArtificialtight junction antagonist peptide 4Gly Arg Val Leu Val Gln Asp Gly1
558PRTArtificialtight junction antagonist peptide 5Gly Arg Leu Cys Val Gln Pro Gly1
568PRTArtificialtight junction antagonist peptide 6Gly Arg Leu Cys Val Gln Asp Gly1
578PRTArtificialtight junction antagonist peptide 7Gly Arg Leu Leu Val Gln Pro Gly1
588PRTArtificialtight junction antagonist peptide 8Gly Arg Leu Leu Val Gln Asp Gly1
598PRTArtificialtight junction antagonist peptide 9Gly Arg Gly Cys Val Gln Pro Gly1
5108PRTArtificialtight junction antagonist peptide 10Gly Arg Gly Cys Val Gln Asp Gly1
5118PRTArtificialtight junction antagonist peptide 11Gly Arg Gly Leu Val Gln Pro Gly1
5128PRTArtificialtight junction antagonist peptide 12Gly Arg Gly Leu Val Gln Asp Gly1
5138PRTArtificialtight junction antagonist peptide 13Gly Gly Val Cys Val Gln Pro Gly1
5148PRTArtificialtight junction antagonist peptide 14Gly Gly Val Cys Val Gln Asp Gly1
5158PRTArtificialtight junction antagonist peptide 15Gly Gly Val Leu Val Gln Pro Gly1
5168PRTArtificialtight junction antagonist peptide 16Gly Gly Val Leu Val Gln Asp Gly1
5178PRTArtificialtight junction antagonist peptide 17Gly Gly Leu Cys Val Gln Pro Gly1
5188PRTArtificialtight junction antagonist peptide 18Gly Gly Leu Cys Val Gln Asp Gly1
5198PRTArtificialtight junction antagonist peptide 19Gly Gly Leu Leu Val Gln Pro Gly1
5208PRTArtificialtight junction antagonist peptide 20Gly Gly Leu Leu Val Gln Asp Gly1
5218PRTArtificialtight junction antagonist peptide 21Gly Gly Gly Cys Val Gln Pro Gly1
5228PRTArtificialtight junction antagonist peptide 22Gly Gly Gly Cys Val Gln Asp Gly1
5238PRTArtificialtight junction antagonist peptide 23Gly Gly Gly Leu Val Gln Pro Gly1
5248PRTArtificialtight junction antagonist peptide 24Gly Gly Gly Leu Val Gln Asp Gly1
52520PRTHomo sapiens 25Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly1 5 10
15Ser Leu Arg Leu 20269PRTHomo sapiens 26Val Thr Phe Tyr Thr Asp Ala Val Ser1 52720PRTHomo
sapiensmisc_feature(16)..(16)Xaa can be any naturally occurring amino acid 27Met Leu Gln Leu Ala Glu
Ser Gly Gly Val Leu Val Gln Pro Gly Xaa1 5 10 15Ser Asp Arg Leu 202811PRTHomo
sapiensmisc_feature(10)..(10)Xaa can be any naturally occurring amino acid 28Glu Val Gln Leu Val Glu
Ser Gly Gly Xaa Leu1 5 10298PRTArtificialtight junction antagonist peptide 29Val Gly Val Leu Gly Arg
Pro Gly1 5308PRTArtificialtight junction antagonist peptide 30Val Asp Gly Phe Gly Arg Ile Gly1
53122PRTHomo sapiensmisc_feature(1)..(1)Xaa can be any naturally occurring amino acid 31Xaa Gly
Leu Val Leu Val Gly Val Asn Gly Phe Gly Arg Ile Gly Arg1 5 10 15Ile Gly Arg Leu Val Ile
203220PRTHomo sapiens 32Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Arg1 5 10
15Ser Leu Arg Leu 203314PRTVibrio cholerae 33Phe Cys Ile Gly Arg Leu Cys Val Gln Asp Gly Phe
Val Thr1 5 10

Read more: <http://www.faqs.org/patents/app/20080269136#ixzz0RyIfpYsh>

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1: Scand J Gastroenterol. 2006 Apr;41(4):408-19. Links

Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines.

Drago S, El Asmar R, Di Pierro M, Grazia Clemente M, Tripathi A, Sapone A, Thakar M, Iacono G, Carroccio A, D'Agate C, Not T, Zampini L, Catassi C, Fasano A.

Mucosal Biology Research Center, Center for Celiac Research and Division of Pediatric Gastroenterology and Nutrition, University of Maryland, School of Medicine, Baltimore, MD 21201, USA.

ESSENCE OF ARTICLE

“CONCLUSIONS: Based on our results, we concluded that gliadin activates zonulin signaling irrespective of the genetic expression of autoimmunity, leading to increased intestinal permeability to macromolecules.”

ARTICLE

OBJECTIVE: Little is known about the interaction of gliadin with intestinal epithelial cells and the mechanism(s) through which gliadin crosses the intestinal epithelial barrier. We investigated whether gliadin has any immediate effect on zonulin release and signaling. **MATERIAL AND METHODS:** Both ex vivo human small intestines and intestinal cell monolayers were exposed to gliadin, and zonulin release and changes in paracellular permeability were monitored in the presence and absence of zonulin antagonism. Zonulin binding, cytoskeletal rearrangement, and zonula occludens-1 (ZO-1) redistribution were evaluated by immunofluorescence microscopy. Tight junction occludin and ZO-1 gene expression was evaluated by real-time polymerase chain reaction (PCR). **RESULTS:** When exposed to gliadin, zonulin receptor-positive IEC6 and Caco2 cells released zonulin in the cell medium with subsequent zonulin binding to the cell surface, rearrangement of the cell cytoskeleton, loss of occludin-ZO1 protein-protein interaction, and increased monolayer permeability. Pretreatment with the zonulin antagonist FZI/0 blocked these changes without affecting zonulin release. When exposed to luminal gliadin, intestinal biopsies from celiac patients in remission expressed a sustained luminal zonulin release and increase in intestinal permeability that was blocked by FZI/0 pretreatment. Conversely, biopsies from non-celiac patients demonstrated a limited, transient zonulin release which was paralleled by an increase in intestinal permeability that never reached the level of permeability seen in celiac disease (CD) tissues. Chronic gliadin exposure caused down-regulation of both ZO-1 and occludin gene expression. **CONCLUSIONS:** Based on our results, we concluded that gliadin activates zonulin signaling irrespective of the genetic expression of autoimmunity, leading to increased intestinal permeability to macromolecules.

[HTTP://JN.NUTRITION.ORG/CGI/CONTENT/ABSTRACT/139/4/710?HITS=&SORTSPEC=RELEVANCE&HITS=&FDATE=%2F%2F&ANDOREXACTTITLE=OR&MAXTOSHOW=&ANDOREXACTFULLTEXT=AND&FIRSTINDEX=0&FULLTEXT=+TIGHT+JUNCTION++CACO+2+WESTERN+BLOT&RESOURCETYPE=HWCIT&SEARCHID=1&RESULTFORMAT=1](http://jn.nutrition.org/cgi/content/abstract/139/4/710?hits=&sortspec=relevance&hits=&fdate=%2F%2F&andorexacttitle=or&maxtoshow=&andorexactfulltext=and&firstindex=0&fulltext=+tight+junction++caco+2+western+blot&resourcetype=hwcit&searchid=1&resultformat=1)

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Nutrition and Disease

Glutamine Deprivation Alters Intestinal Tight Junctions via a PI3-K/Akt Mediated Pathway in Caco-2 Cells^{1,2}

Nan Li and Josef Neu*

Department of Pediatrics, University of Florida College of Medicine, Gainesville, FL 32610

ESSENCE OF ARTICLE

“In conclusion, Gln regulates intercellular junction integrity and TJ proteins through the PI3-Kinase/Akt pathway. “

ARTICLE

Glutamine (Gln) is important for intestinal barrier function and regulation of tight junction (TJ) proteins, but the intracellular mechanisms of action remain undefined. The purpose of this study was to test the hypothesis that Gln regulates intercellular junction integrity and TJ proteins through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in Caco-2 cells. Deprivation of exogenous and endogenous glutamine decreased transepithelial electrical resistance (TER) ($P < 0.01$) and increased permeability ($P < 0.01$). Both wortmannin and LY294002, PI3K inhibitors, prevented the TER decrease and the permeability increase induced by Gln deprivation ($P < 0.001$). Gln deprivation also caused decreased TJ protein claudin-1 ($P < 0.001$). Both wortmannin and LY294002 treatment prevented this effect ($P < 0.001$). Deprivation of Gln increased phosphor-Akt protein. Gln supplementation reversed this effect. Decreased TER and increased permeability associated with Gln deprivation were not observed in small interfering RNA for p85 transfected Caco-2 cells. In conclusion, Gln regulates intercellular junction integrity and TJ proteins through the PI3-Kinase/Akt pathway.

* To whom correspondence should be addressed. E-mail: neuj@peds.ufl.edu .

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MUCOSAL BIOLOGY

Glutamine regulates Caco-2 cell tight junction proteins

Nan Li,¹ Patricia Lewis,² Don Samuelson,² Kellym Liboni,¹ and Josef Neu¹

1Department of Pediatrics, 2Small Animal Clinic Science, University of Florida College of Medicine, Gainesville, Florida 32610

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ESSENCE OF ARTICLE

”These findings indicate that TJ protein expression and cellular localization in Caco-2 cell monolayers rely on GLN. This mechanism may similarly relate to GLN-mediated modulation of intestinal barrier function in stressed animals and humans. “

ARTICLE

ABSTRACT

Intestinal epithelial tight junction (TJ) barrier dysfunction may lead to inflammation and mucosal injury. Glutamine (GLN) plays a role in maintenance of intestinal barrier function in various animal models and critically ill humans. Recent evidence from intestinal cell monolayers indicates that GLN maintains transepithelial resistance and decreases permeability. The mechanisms of these effects remain undefined. We hypothesized that GLN affects proteins involved in the intercellular junctional complex. GLN availability was controlled in Caco-2 monolayers by addition to the medium and treatment with methionine sulfoximine (MSO) to inhibit glutamine synthetase (GS). Expression of TJ proteins, claudin-1, occludin, and zonula occluden (ZO)-1 was measured by immunoblotting. Localization of TJ proteins was evaluated by immunofluorescence light microscopy. Structure of TJ was determined by transmission electron microscopy (TEM). Deprivation of GLN decreased claudin-1, occludin, and ZO-1 protein expression and caused a disappearance of perijunctional claudin-1 and a reduction of occludin but had no effect on ZO-1. TEM revealed that MSO-treated cells in the absence of GLN formed irregular junctional complexes between the apical lateral margins of adjoining cells. These findings indicate that TJ protein expression and cellular localization in Caco-2 cell monolayers rely on GLN. This mechanism may similarly relate to GLN-mediated modulation of intestinal barrier function in stressed animals and humans.

nutrition; intercellular junctions; intestinal epithelia cells

IN ADDITION TO BEING THE ORGAN responsible for digestion and absorption of nutrients, the intestine serves a barrier function that is a critical component of the innate immune system (2, 3, 8, 12). Only a single layer of epithelial cells separates the luminal contents from effector immune cells in the lamina propria and the internal milieu of the body. Breaching this single layer of epithelium can lead to pathological exposure of the highly immunoreactive subepithelium to the vast number of microbes and antigens in the lumen. Breakdown of the barrier is implicated in bacterial translocation, leading to sepsis, and in the pathogenesis of acute illnesses such as multiple organ system failure (22). Increased permeability early in life has been implicated in the pathogenesis of several diseases that manifest in later life, including atopy, food allergies, celiac enteropathy, type 1 diabetes, inflammatory bowel disease (2, 8), and autism (39). Studies in rodents show that stresses such as physical restraint can lead to increased

ileal permeability (27), and early separation of the mother from the infant is a stress that is associated with intestinal permeability disorders later in life (35).

The mechanisms of stress-related epithelial breakdown are unclear, but regulation of paracellular pathways, especially via the interepithelial tight junction (TJ) proteins, and subsequent stimulation of highly immunoreactive submucosal cells are likely to play a significant role. The TJ represents the major barrier within the paracellular pathway between intestinal epithelial cells (14). These are dynamic structures that readily adapt to a variety of developmental (23, 34), physiological (24), and pathological (28, 29) circumstances. These adaptive mechanisms are still incompletely understood. Multiple proteins that make up the TJ have been identified: occludin (10) and members of the claudin family (9), a group of at least 20 tissue-specific proteins, are the major sealing proteins (36). Studies of occludin "knockout" mice have demonstrated that they retain normal intestinal permeability and, despite demonstrating poor growth and other phenotypic abnormalities, remain viable (33). Recent studies suggest that claudin-1, the intestine-associated family member (17), may directly associate with occludin laterally in the membrane within the same cell but not intercellularly (11), and the combination of these two proteins functioning together performs the major "gatekeeper" function of the TJ (14). These sealing proteins, both transmembrane proteins, interact with cytoplasmic plaques that consist of different types of cytosolic proteins (including the zonula occludens proteins ZO-1, ZO-2, and ZO-3).

Very little is known about specific nutrients and how they affect intestinal epithelial junctions. Evidence suggests that glutamine (GLN) helps maintain intestinal mucosal integrity, especially during stresses, such as radiation therapy (4), chemotherapy (1), and total parenteral nutrition (20), and blunts increased gut permeability associated with experimental sepsis (7).

Of the *in vitro* models, Caco-2 cells are one of the most widely used to study the assembly of intestinal intercellular junctions and the development of apical-basolateral polarity during differentiation. Caco-2 cells grow to confluence and spontaneously differentiate in a process requiring 21 days (25). Depriving Caco-2 cell monolayers of GLN leads to increased bacterial translocation (31). Previous studies from our laboratory have demonstrated that deprivation of GLN from cell culture medium and inhibition of glutamine synthetase (GS) using methionine sulfoximine (MSO) lead to significant decreases in transepithelial resistance of Caco-2 cell monolayers and increased permeability (6, 38). In the absence of added GLN, sufficient GLN may be provided *de novo* by GS. Similar results have been found in a cell culture stress model (19). Electron microscopy of the intestine in a GLN-deprived infant rat model also demonstrates intestinal intercellular junction breakdown (32). The barrier function breakdown in Caco-2 cells is reversible, suggesting that it is not the result of permanent cell damage (6). These studies establish a role for GLN, either from the medium or via endogenous synthesis, in supporting epithelial cell barrier function of the monolayers.

A relationship between a single nutrient, cell junction biophysical properties, paracellular permeability, and TJ proteins has not been established previously. To test the hypothesis that Caco-2 intercellular junction TJ proteins are involved in the transepithelial resistance and permeability seen with GLN deprivation, immunoblotting, immunofluorescence light microscopy, and transmission electron microscopy were performed.

MATERIALS AND METHODS

Reagents. Vendor-prepared solutions of Trypsin/EDTA, MEM, FBS, and antibiotic antimycotic solution were from GIBCO-BRL (Grand Island, NY). Biocoat Cell Culture Inserts (Fibrillar Collagen, type I rat tail) and MITO+ Serum Extender were from Collaborative Biomedical Products (Bedford, MA). MSO [L-S-(3-amino-3-carboxypropyl)-S-methylsulfoximine], L-GLN, and all other chemical reagents were from Sigma Chemical (St. Louis, MO). Antibodies (anti-claudin-1, occludin, and ZO-1) were obtained from Zymed Laboratories (San Francisco, CA).

Cell culture. Caco-2 cells were purchased from ATCC (Rockville, MD) and grown in a humidified incubator at 37°C with 5% CO₂ and 95% air. Cells between passage 20 and 30 were used for all experiments. For each experiment, cells were collected by dissociation of a confluent stock culture with 0.05% trypsin and 0.53 mM EDTA, counted using a hemacytometer. Cells were seeded in 24-well Biocoat Cell Culture Inserts (Collaborative Biomedical Products) at 200,000 cells/well for electron microscopy, Nunc flasks (Nalge Nunc International, Naperville, IL) for immunoblotting, or Lab-Tek II Chamber Slide (Nalge Nunc International) for immunofluorescence microscopy. Culture media consisted of 8:2 GLN-free MEM and FBS. Cells were cultured with 4 mM GLN for 21 days before treatment. Next, cells were fed with GLN-free DMEM with MITO+ Serum Extender (Collaborative Biomedical Products), a substitute of serum, including hormones, growth factors, and defined metabolites, but not GLN. The medium was supplemented with the indicated concentration of GLN for various time points. Media was changed every other day. Media also contained penicillin, streptomycin, and amphotericin B at concentrations of 200 U/ml, 200 µg/ml, and 0.05 µg/ml, respectively.

Preparation of detergent-soluble and -insoluble protein fractions. Caco-2 cells were washed three times with ice-cold PBS, immediately incubated in Nonidet P-40 extraction buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 25 mM NaF, 1 mM Na₃VO₄, 10 mM sodium pyrophosphate, and protease inhibitors) on ice for 30 min, and centrifuged at 20,000 g for 30 min at 4°C. The detergent-soluble fraction was transferred to a microcentrifuge tube. The insoluble fraction was collected in SDS extraction buffer (25 mM HEPES, pH 7.4, 4 mM EDTA, 1% SDS, 25 mM NaF, 1 mM Na₃VO₄, and 10 mM sodium pyrophosphate) and sonicated. Supernatants (cytoskeleton-associated fractions) were then obtained after centrifugation (20,000 g for 30 min) and diluted with an equal volume of Nonidet P-40 extraction buffer to reduce SDS concentration in the samples. Protein concentrations were measured using a Bio-Rad Dc Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) according to the instructions.

Western blotting. Equal amounts of proteins (20 µg) for each sample were separated on SDS-PAGE (12.5% polyacrylamide gel) and transferred to Immobilon Transfer polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were blocked with 5% nonfat milk, incubated sequentially with primary antibody (1:2,000 for claudin-1, 1:1,000 for ZO-1 and occludin) and horseradish peroxidase-conjugated secondary antibody (1:2,000), and detected with ECL plus (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were quantified by densitometry using Adobe Photoshop software.

Immunofluorescence microscopy. Cells (4 x 10⁵ cells/cm²) were grown in the Lab-Tek II chamber slide system (Nalge Nunc International) with various treatments. After being washed with PBS (without Ca²⁺ and Mg²⁺), cells were fixed with 3% paraformaldehyde. Primary antibody claudin-1, occludin, or ZO-1 (Zymed Laboratories, San Francisco, CA) was diluted 1:50 and incubated for 1 h. Next, cells were incubated with a secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG

antibody; Sigma] for 30 min at 1:100 dilutions. After being mounted with FITC-compatible media, TJ protein expression was visualized under a fluorescent light microscope (Leitz).

Transmission electron microscopy. After dehydration and embedding in Araldite epoxy resin, 1- μ m-thick sections were made using glass knives on a Reichert Ultracut S ultramicrotome and stained with 1% toluidine blue in 1% sodium borate. Areas selected for ultrastructural observations and ultrathin sections (80 nm in thickness) were cut using a diamond knife and stained with saturated uranyl acetate and Reynold's lead citrate. Sections were viewed on a Hitachi H7000 transmission electron microscope, and images were captured digitally. Ultrastructure observations were made from multiple sites (>10) of junctional complexes that were clearly identified. At least three images from each treatment group were analyzed by three people in a blinded fashion.

Statistical analysis. Sigmastat statistical software (SPSS, Chicago, IL) was used to analyze densitometry results of TJ protein relative expression levels for Western blots. All data were reported as means \pm SD from three independent experiments. A one-way ANOVA was used to determine if a significant difference was present among all treatment groups. Additionally, Bonferroni t-tests were performed for unpairwise comparisons when the ANOVA was significant at $P < 0.05$.

RESULTS

Effects of GLN on claudin-1, occludin, and ZO-1 protein expression. To further determine if TJ-associated proteins are involved in the GLN-mediated effects on Caco-2 cell intercellular junctions, Caco-2 cells were cultured for 21 days and treated with or without GLN for 5 days. Detergent-soluble and -insoluble protein fractions were isolated from the cells. Western blots were done to evaluate the expression levels of TJ proteins.

Using an antibody specific for claudin-1, no differences were seen in the detergent-soluble proteins among the different doses of GLN, whereas deprivation of GLN decreased the claudin-1 content and GLN restored claudin-1 expression in a dose-dependent manner when examined in the detergent-insoluble fractions ($P < 0.05$ vs. GLN 0 group; Fig. 1A).

Fig. 1. Effects exogenous glutamine (GLN) on tight junction proteins. Caco-2 cells were cultured for 3 wk and treated with indicated concentration of GLN for 5 days. Detergent-soluble (S) and -insoluble (I) protein fractions were isolated from the cells. Protein samples were subjected to SDS-PAGE. Western blots were probed with anti-claudin-1 (A), anti-occludin (B), and anti-zonula occluden (ZO)-1 (C) antibodies. Densitometry results were shown in the corresponding bar graphs for relative expression level of claudin-1, occludin, and ZO-1. The protein expression level in GLN-free group was 100%. All data were reported as means \pm SD from 3 independent experiments. * $P < 0.05$ vs. GLN-free group.

The higher molecular form, a phosphorylated form (85 kDa) of occludin, is associated with the assembling of TJ complexes. In contrast, the low molecular form, a nonphosphorylated form (65 kDa), is involved in the disassembling of TJs (13). As shown in Fig. 1B, both 85- and 65-kDa forms were seen when detergent-insoluble protein extracts were examined. However, the 65-kDa form was predominant in the

detergent-soluble fraction. GLN did not affect 65-kDa occludin expression in either detergent-soluble or -insoluble fractions (Fig. 1B). Likewise, Fig. 1C shows no change of ZO-1 expression in either detergent-soluble or -insoluble protein with different doses of GLN. These results suggest that GLN provided in the culture medium (exogenous GLN) regulates claudin-1 protein level in the detergent-insoluble form, but not occludin or ZO-1.

To investigate the role of GLN synthesized intracellularly via GS (endogenous GLN) in regulating TJ protein, Caco-2 cells were cultured as above and treated with or without GLN in the absence or presence of 4 mM MSO, a specific inhibitor of GS. As shown in Fig. 2, claudin-1 expression level was the lowest when there was no GLN in the medium, and GS was also inhibited by MSO, especially in the detergent-insoluble fraction ($P < 0.01$ vs. GLN 0/MSO 0 group; Fig. 2A). GLN supplementation rescued the decrease of claudin-1 caused by GLN deprivation in a dose-dependent manner (Fig. 2, A and D). Endogenous (synthesized de novo via GS) and exogenous (supplied in the media) GLN deprivation caused a decrease of occludin expression in both detergent-soluble and -insoluble fractions ($P < 0.05$ vs. GLN 0/MSO 0 group; Fig. 2B). Meanwhile, GLN supplementation restored the occludin content even at a very low dose (0.1 mM) of GLN (Fig. 2B). Similarly, ZO-1 was reduced by GLN deprivation (Fig. 2C). GLN (4 mM) increased the ZO-1, especially in detergent-insoluble fractions ($P < 0.05$ vs. GLN 0/MSO 0 group; Fig 2C). These results demonstrate that endogenous GLN also plays an important role in regulation of TJ proteins.

Fig. 2. Effect of endogenous GLN on tight junction proteins. Caco-2 cells were treated as above and in the absence or presence of 4 mM methionine sulfoximine (MSO). Detergent-soluble and -insoluble protein fractions were isolated from the cells. Protein samples were subjected to SDS-PAGE. Western blots were probed with anti-claudin-1 (A), anti-occludin (B), and anti-ZO-1 (C) antibodies. Densitometry results were shown in the corresponding bar graphs for relative expression level of claudin-1, occludin, and ZO-1. The protein expression level in GLN 0/MSO 0 group was 100%. All data were reported as means \pm SD from 3 independent experiments. * $P < 0.05$ vs. GLN 0/MSO 0 group. ** $P < 0.01$ vs. GLN 0/MSO 0 group.

Figure 3 shows the time course of the effects of GLN deprivation on TJ protein expression. Caco-2 cells were treated with GLN-free medium in the presence of MSO at the indicated time points. Cells were treated with 0.6 mM GLN in the presence of MSO for 48 h as a control. In the detergent-insoluble fraction, GLN deprivation downregulated claudin-1, and the maximum effect was at 108 h ($P < 0.05$; Fig. 3A). In the detergent-soluble fraction, there appeared to be a trend toward decrease of claudin-1 from 48 h with a maximum effect at 108 h, but there is no statistical significance ($P > 0.05$; Fig. 3A). GLN deprivation caused a decrease in occludin in the detergent-soluble fraction from 48 to 108 h and in insoluble fractions at 108 h ($P < 0.05$; Fig. 3B) and had a similar effect on ZO-1 in insoluble fractions at 108 h ($P < 0.05$; Fig. 3C).

Fig. 3. Time course of tight junction expression. Caco-2 cells were treated with GLN-free medium with 4 mM MSO, a glutathione synthase (GS) inhibitor. Cells were harvested at the indicated time points. Western blotting was done to detect the expression levels of claudin-1 (A), occludin (B), and ZO-1 (C) in Caco-2 cells. Cells were treated with 0.6 mM GLN in the presence of 4 mM MSO as a control. Densitometry results were shown in the corresponding bar graphs for relative expression level of

claudin-1, occludin, and ZO-1. The protein expression level in GLN 0.6 mM/MSO 4 mM 48-h group (control) was 100%. All data were reported as means \pm SD from 3 independent experiments. *P < 0.05 vs. control group.

Effects of GLN on TJ protein localization (fluorescence microscopy). To test if GLN affects the localization of TJ proteins, cells were treated as above. Immunofluorescence was done by using fluorescent-labeled antibodies to occludin, claudin-1, and ZO-1. Cell morphology among the different treatment groups appeared to differ, with the GLN 0/MSO 4 mM cells appearing slightly larger than the others (Fig. 4, A-D). The greatest GLN depletion (no GLN in the medium and endogenous GLN synthesis inhibited with MSO) indicated that claudin-1 (Fig. 4G) and occludin (Fig. 4K) were markedly decreased (intercellular junctions of cells lose bright green, well-defined outline). GLN deprivation did not obliterate junctions in the ZO-1 immunolabeled cells (Fig. 4, M and O) as it did with occludin and claudin-1, suggesting a different effect of GLN on the individual TJ proteins.

Fig. 4. Effects of GLN on tight junction protein localization using immunofluorescence. Caco-2 cells were treated with (B, D, F, H, J, L, N, and P) or without (A, C, E, G, I, K, M, and O) 0.6 mM GLN in the absence (A, B, E, F, I, J, M, and N) or presence (C, D, G, H, K, L, O, and P) of MSO for 5 days. Cells were fixed and observed under light microscope (A–D). Immunofluorescence was done by using specific antibodies for claudin-1 (E–H), occludin (I–L), and ZO-1 (M–P) and observed under a fluorescence microscope. Magnification was x400.

Effects of GLN on ultrastructure of TJ. Ultrastructure of Caco-2 cells treated as previously described was shown by transmission electron microscopy (Fig. 5). The presence of electron-dense material in the space between cells near the brush border reflects the TJ. In cells with 0.6 mM GLN (Fig. 5B), the TJ and desmosome displayed an intact structure. In cells without GLN and in the absence of MSO in the medium, the TJ and desmosome appear preserved (Fig. 5A). When the cells are deprived of exogenous and de novo synthesized GLN (with 4 mM MSO; Fig. 5C), the TJ complex appeared reduced and contained less electron-dense material, whereas the desmosomes were still evident. With 0.6 mM GLN, this effect was rescued (Fig. 5D). These results demonstrated that GLN deprivation resulted in distortion of normal TJ morphology.

Fig. 5. Effects of GLN on ultrastructure of tight junctions. Caco-2 cells were treated with (B and D) or without (A and C) 0.6 mM GLN in the absence (A and B) or presence (C and D) of MSO for 5 days. Cells were processed for transmission electron microscopy (TEM) as described in MATERIALS AND METHODS. The junction structures between the adjoining cells were observed under TEM. Arrows, tight junctions; arrowheads, desmosomes. Bars = 500 nM.

DISCUSSION

GLN helps maintain intestinal mucosal integrity, especially during stresses, such as radiation therapy (4), chemotherapy (1), and total parenteral nutrition (20), and blunts increased gut permeability associated with experimental sepsis (7). Previous studies from our laboratory demonstrate that, when endogenous GS is inhibited by MSO in addition to nutritional deprivation of GLN, Caco-2 culture systems exhibit

significant breakdown in barrier function (6). Caco-2 cell models subjected to media change stress (19) or "luminal starvation" (18) wherein inhibition of GS was not required for breakdown in barrier function also responded to GLN by decreasing permeability. It has also been shown that GLN deprivation caused a breakdown of the epithelial junctions in a gastrostomy-fed infant rat model (32).

The Western immunoblots demonstrate that deprivation decreases the insoluble fraction of claudin-1 and occludin and that this effect is partially reversible with GLN supplementation. This is supported by the immunofluorescence studies showing that both occludin and claudin-1 are affected by GLN depletion. To test if GLN 0/MSO 4 mM could cause cell death, lactate dehydrogenase (LDH) assays were done to evaluate cell viability. Cells were treated as above. There was no difference in percentage of LDH release among various GLN/MSO treatment groups (data not shown). This result demonstrated that GLN deprivation does not cause the cell death, and GLN deprivation has a specific effect on TJ protein. The fact that ZO-1 protein was affected on the protein immunoblot but not demonstrated in the immunofluorescent microscopy is of interest. It is notable that the cells in the GLN-depleted group where ZO-1 was examined appeared larger, but we do not know how this might relate to the discrepancy between decreased ZO-1 protein in the immunoblots and normal discrete junctions in the immunofluorescence studies. The electron microscopy supports the protein immunoblot data by demonstrating a poorly visualized TJ in the GLN-deprived group compared with the GLN-supplemented groups or the group that was allowed to synthesize GLN via GS (Fig. 5).

Several stressors have been found to have profound effects on intestinal TJ. These include bacterial toxins (30), commonly used drugs such as indomethacin (30), and allergens including gliadin (5). To our knowledge, the current studies are the first to establish a relationship between intestinal cell GLN status and intercellular junction proteins. The fact that claudin-1 and occludin were affected by GLN is of interest. The claudin family consists of 15 closely related proteins, several of which have been found in distinct tissues and defects of which have been implicated in several disease processes involving paracellular permeability (16, 37). Claudin-1 was chosen for evaluation in this study primarily because of its previous association with intestinal epithelial cells (41). Claudin and occludin appear to work in concert and appear to be necessary for normal paracellular function (14, 15).

Although these studies demonstrate a relationship between TJ proteins and paracellular biophysical, physiological properties, and intestinal epithelial GLN status, the mechanisms of these effects remain unclear. Although the possibility exists that the decrease in TJ proteins is simply the result of altered protein synthesis, the overall TJ protein concentrations did not differ on the Western blots, whereas the greatest effect was seen in the detergent-insoluble fractions, which suggests a more direct effect on TJ protein localization. This conclusion was validated by both light and electron microscopy in these studies.

There currently exist several candidate pathways that might be related to nutrition and stress-related signaling mechanisms of TJ assembly (26). Several lines of evidence have implicated phosphatidylinositol 3-kinase (PI3K) in regulating TJ assembly. Previous investigators have found that prostaglandins stimulate recovery of paracellular resistance via a mechanism involving transepithelial osmotic gradients and PI3K-dependent restoration of TJ protein distribution (21). These findings suggest that the PI3K, phosphatidylinositols, and filamentous actin rearrangements, in combination, play an important role in the modulation of the junction integrity.

In addition to PI3K, two Rho family GTPases, Rho and Rac, have also emerged as key regulators acting antagonistically to regulate endothelial barrier function: Rho increases actin-myosin contractility, which facilitates breakdown of intercellular junctions, whereas Rac stabilizes endothelial junctions and counteracts the effects of Rho (40). Similar mechanisms (Rho and Rac) are implicated in the regulation of intestinal epithelial junction integrity. Whether GLN affects either PI3K or the Rho/Rac pathways remains speculative.

In summary, GLN status affects TJ proteins in a manner that is commensurate to its effects on electrical resistance and permeability. Further evaluation of nutritional agents affecting these proteins and their mechanism of action will be critical because they have major implications in the pathogenesis of several disease entities.

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FOOTNOTES

Address for reprint requests and other correspondence: J. Neu, Division of Neonatology, Dept. of Pediatrics, Univ. of Florida, College of Medicine, P.O. Box 100296, Gainesville, FL 32610 (E-mail: neuj@peds.ufl.edu)

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Biochemical, Molecular, and Genetic Mechanisms

Butyrate Enhances the Intestinal Barrier by Facilitating Tight Junction Assembly via Activation of AMP-Activated Protein Kinase in Caco-2 Cell Monolayers^{1,2}

Luying Peng^{3,5}, Zhong-Rong Li⁴, Robert S. Green³, Ian R. Holzman³ and Jing Lin^{3,*}

³ Department of Pediatrics, Mount Sinai School of Medicine, New York, NY 10029-6574; ⁴ Department of Pediatric Surgery, Yuying Children's Hospital of Wenzhou Medical College, Wenzhou, China 325027; and ⁵ Department of Medical Genetics, Tongji University School of Medicine, Shanghai, China 20009

ESSENCE OF ARTICLE

“We conclude that butyrate enhances the intestinal barrier by regulating the assembly of tight junctions.”

ARTICLE

Butyrate, one of the SCFA, promotes the development of the intestinal barrier. However, the molecular mechanisms underlying the butyrate regulation of the intestinal barrier are unknown. To test the hypothesis that the effect of butyrate on the intestinal barrier is mediated by the regulation of the assembly of tight junctions involving the activation of the AMP-activated protein kinase (AMPK), we determined the effect of butyrate on the intestinal barrier by measuring the transepithelial electrical resistance (TER) and inulin permeability in a Caco-2 cell monolayer model. We further used a calcium switch assay to study the assembly of epithelial tight junctions and determined the effect of butyrate on the assembly of epithelial tight junctions and AMPK activity. We demonstrated that the butyrate treatment increased AMPK activity and accelerated the assembly of tight junctions as shown by the reorganization of tight junction proteins, as well as the development of TER. AMPK activity was also upregulated by butyrate during calcium switch-induced tight junction assembly. Compound C, a specific AMPK inhibitor, inhibited the butyrate-induced activation of AMPK. The facilitating effect of butyrate on the increases in TER in standard culture media, as well as after calcium switch, was abolished by

compound C. We conclude that butyrate enhances the intestinal barrier by regulating the assembly of tight junctions. This dynamic process is mediated by the activation of AMPK. These results suggest an intriguing link between SCFA and the intracellular energy sensor for the development of the intestinal barrier.

* To whom correspondence should be addressed. E-mail: jing.lin@mssm.edu .

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