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Colostrinin Decreases Hypersensitivity and Allergic Responses to Common Allergens

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Key Words
Colostrinin · Immunoglobulin E · Allergic inflammation

Abstract
Background: Colostrinin™ (CLN), isolated from mothers' pre-milk fluid (colostrum), is a uniform mixture of low-molecular-weight, proline-rich polypeptides. CLN induces neurite outgrowth of pheochromocytoma cells, extends the lifespan of diploid fibroblast cells, inhibits β-amyloid-induced apoptosis and improves cognitive functions when administered to Alzheimer's disease patients. ***Objective:*** The aim of this study was to investigate potential allergic responses to CLN and its impact on allergic sensitization and inflammation caused by common allergens. ***Methods:*** We used a well-characterized mouse model of allergic airway inflammation. Changes in IgE/IgG1 and mucin levels, airway eosinophilia and hyperreactivity to methacholine were determined by ELISA, differential cell counting and whole-body plethysmography, respectively. ***Results:*** CLN did not increase IgE/IgG1 levels or induce cutaneous hypersensitivity reaction, airway inflammation and mucin production. Importantly, CLN significantly ($p < 0.001$) decreased IgE/IgG1 production, airway eosinophilia, mucin production and hypersensitivity induced by allergenic extracts from ragweed pollen grains and house dust mites. ***Conclusion:*** CLN itself is non-allergenic; however, it is effective in preventing allergic responses to known indoor and outdoor allergens. These data support the safe application of CLN and its potential use in the prevention of allergic inflammation in humans.

Introduction

Colostrinin™ (CLN) is a uniform complex of small-molecular-weight peptides (3–15 kDa) characterized by high proline content and a high proportion of non-polar amino acids. CLN is purified from pre-milk fluid (colostrum) collected from mothers during the first 24 h after giving birth [1–5]. It has been shown that CLN is an important immune modulator that induces maturation and differentiation of murine thymocytes [3, 6], promotes peripheral blood leukocyte proliferation and induces cytokine production [3, 7]. CLN decreases intracellular oxidative stress levels, reduces 4-hydroxynonenal-mediated cellular damage and suppresses 4-hydroxynonenal-induced cellular signaling in cultured cells [8]. Most importantly, CLN induces delicate cassettes of signaling path-

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ways common to cell proliferation and differentiation and mediates cellular activities that are similar to those of hormones and neurotrophins, leading to neurite outgrowth [9]. CLN protects neuroblastoma cells from β-amyloid-induced apoptosis by inhibiting amyloid aggregation [10]. CLN delays the onset of proliferative senescence of diploid murine fibroblast cells [11]. Moreover, CLN administration to Alzheimer's disease patients has resulted in stabilization of cognitive functions and improvement in the ability to perform routine domestic functions [12–14].

Allergenic proteins are processed by dendritic cells, and their peptide segments are presented in the context of class II major histocompatibility complex molecules to naïve T helper (Th) cells [15]. These interactions orchestrate a cascade that leads to the development of type 2 Th cells (Th2) and consequently the initiation of IgE synthesis [15, 16]. The majority of the allergic reactions are mediated by IgE antibodies, although non-IgE-mediated immune mechanisms may also cause some hypersensitivity disorders [17]. The clinical manifestation of food- and dietary supplement-induced allergic reactions is frequently consistent with the leading categories of human hypersensitivity. Severe reactions to dietary supplements and milk are often associated with respiratory failure, and patients with asthma have a greater risk of severe reactions [17–19]. Coexisting food allergy and allergic airway inflammation is a significant problem in patient populations of varying ages [20].

CLN is being considered a dietary supplement and is being tested in clinical trials to treat/prevent age-related disorders of the central nervous system. Therefore, it is of considerable importance to define whether it can induce allergic reactions and to test its ability to modulate allergen-induced inflammation. To evaluate such potential effects of CLN, we used a mouse model of asthma, which was previously validated in our laboratory [21–23]. In this study, we show for the first time that the oral, intranasal and intraperitoneal administration of CLN resulted in no allergic responses. Further, CLN significantly decreased IgE/IgG1 levels, immediate cutaneous reactions and airway inflammation induced by well-characterized allergenic extracts from house dust mite *(Dermatophagoides pteronyssinus* and *D. farinae)* and ragweed *(Ambrosia artemisiifolia)* pollen grains. Therefore, CLN is a very attractive natural substance for use in preventing and/or decreasing allergic responses localized to mucosal surfaces, skin and airways in humans. These data further underline the risk-free application of CLN for prevention and/or treatment of human diseases.

Materials and Methods

Animals and Sensitization

Animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by the UTMB Animal Care and Use Committee. Six-week-old female BALB/c mice, purchased from Jackson Laboratories (Bar Harbor, Me., USA), were used for the studies. Mice were sensitized with alum-mixed CLN, colostrums (COL), ragweed pollen extract (RWE) or house dust mite extract (HDME; 150 μg/mouse) on days 0 and 4, as we have previously described [21, 23]. In selected experiments, mice were pretreated (conditioned) with CLN by exposing animals to aerosolized CLN (1 mg/ml) for 1 h in an aerosol chamber twice a day for 3 days.

Evaluation of Allergic Inflammation

Animals sensitized with test articles were challenged on day 11, and 72 h later, mice were euthanized to collect bronchoalveolar lavage fluids (BALFs) [24]. The trachea was cannulated and lavage was performed with 2 aliquots of 0.6 ml of ice-cold phosphate-buffered saline (PBS). The BALF cells were collected by centrifugation (800 *g* for 10 min) and further resuspended, counted, and 4 microscope slide spreads per BALF preparations were made. Cells were stained with hematoxylin and eosin (H&E). The eosinophils, neutrophils, lymphocytes and macrophages on the stained cytocentrifuge preparations were enumerated by counting at least 400 cells [21, 22]. Four preparations per BALF were coded, and cells were counted by 2 independent observers on 2 separate occasions. The individuals were blinded from their original cell count for each slide. The intra-observer repeatability was confirmed by comparing the differential cell counts performed on 2 occasions by the same observer (same slides). The inter-observer consistency was confirmed by comparing the differential cell counts of both observers. Assessment of lung histology was carried out as previously described [21, 22]. Briefly, following BALF collection, the lungs were fixed by inflating with formalin. Coronal sections of the formalin-fixed lungs were stained with 2 different stains: (1) H&E for assessing inflammation in subepithelial regions, and (2) periodic acid Schiff stain for assessing the abundance of mucin-producing cells. Stained sections were analyzed using a Photometrix CoolSNAP Fx CCD digital camera mounted on a Nikon Eclipse TE 200 fluorescent microscope [21].

Measurement of Mucin Levels

To determine mucin levels, BALF was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were kept at –80°C until assayed. MUC5AC levels in the BALFs were assessed by ELISA [21]. Briefly, serial dilutions of BALFs were incubated at 40°C in triplicate 96-well plates until dry. Plates were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h and incubated with 50 μl (1:10,000 dilutions) of biotic-conjugated mouse monoclonal MUC5AC antibody (Lab Vision, Fremont, Calif., USA). After 60 min incubation, wells were washed with PBS-BSA and further incubated with streptavidin-horseradish peroxidase goat anti-mouse IgG conjugate (1:10,000) for 1 h. Plates were washed (with PBS-BSA, twice) and peroxidase substrate (3,3′,5,5′-tetramethylbenzidine) was added to obtain a colorimetric product, which was quantified at 450 nm. Data were expressed as arbitrary units. MUC5AC dilutions were included on each plate as a positive control [21].

Airway Responsiveness

The changes in pause of breathing (Penh) as an index of airway obstruction was measured by barometric plethysmography using whole-body plethysmography (Buxco Electronics Inc., Troy, N.Y., USA). Bronchial hyperreactivity was evaluated using the methacholine challenges [21, 25]. Briefly, mice were placed in a Buxco chamber, allowed to acclimate for 5 min and then exposed for 3 min to nebulized saline. Subsequently, mice were exposed to increasing concentrations (0, 6.25, 12.5, 25, 50 and 75 mg/ml) of nebulized methacholine (Sigma Chemical) in saline. The median size of the aerosol ranged between 1 and 4 μm (manufacturer's specification). Bronchopulmonary resistance was expressed as enhanced pause = [(expiratory time/relaxation time) – 1] × (peak expiratory flow/peak inspiratory flow). The flow signals and the desired respiratory parameters were calculated using a BioSystem XA program (Buxco Electronics).

Hypersensitivity Skin Test

Antigen-specific, immediate cutaneous reactivity was determined as previously described [26]. Eight-week-old mice were sensitized with alum-mixed test samples, and on day 10, their bellies were shaved. On day 11, 1 μg of test articles in PBS (5 μl volume) was injected intradermally. Test articles were: CLN, COL, RWE and HDME diluted in PBS. Histamine (Sigma, Inc.) was applied as a positive control. A distance of at least 15 mm was kept between the sites of injections to avoid a confluence of reaction wheals. Skin tests were considered positive when the wheal diameter was >4 mm at 20 min after substance injection. Reactions were considered negative when the wheal diameter was ≤3 mm. Skin test responses were evaluated by an investigator who was unaware of the treatment status of the animals.

Quantification of IgE and IgG1 Levels

CLN (COL, HDME and RWE as controls) specific IgE was measured in serum samples by an immunoenzymatic assay [21]. Briefly, microtiter wells were coated with CLN, COL, HDME or RWE and blocked with 1% BSA-PBS. Plates were incubated with serum samples diluted in PBS-BSA for 1 h, washed with PBS-BSA and probed with either biotin-conjugated anti-mouse IgE (Pharmingen, R35-72) or biotin-conjugated anti-mouse IgG1 (Pharmingen, No. 02002D). Plates were developed with avidin-alkaline phosphatase enzyme (Sigma-Aldrich) and pNPP substrate (Sigma-Aldrich). IgE (and IgG1) concentrations in serum samples were determined from a standard curve of mouse IgE or IgG1.

Testing for Adverse Effects of COL and CLN

To ensure that there were no allergic reactions to CLN (and COL from which CLN derived) in experimental animals, we carried out 3 sets of preliminary studies. First, an initial maximum dose (10 mg per animal = 500 mg/kg body mass) of CLN or COL, with or without alum, was administered intraperitoneally. The control group (n = 7) was treated with solvent (PBS). During the 4-week period, there were no changes in food and water consumption, sleeping-social habits and daytime activities between CLN- and COL-treated animals, as well as control (PBS) groups. Next, the effects of chronic CLN addition on alimentary tracks were examined. Mice were fed 1 mg/day (50 mg/kg/day) CLN (or COL) for 2 weeks. During the 4-week observation period of the CLN-fed group (n = 8), we recorded no diarrheagenic activities or changes in food and water consumption, sleeping-social habits or daytime activities. There were no increases in serum levels of IgE/IgG1 specific to CLN. Three out of 9 COL-fed animals showed mild diarrheagenic activity, while food and water consumption and daily activities were not affected. There were only minor increases in serum levels of IgE/IgG1 specific to COL. We have also investigated the effect of CLN after a single intranasal administration of 100 and 200 μg CLN (in 80 μl PBS per non-sensitized animal). Control animals received the same dose of COL or PBS. Seventy-two and 144 h after challenge, BALFs of animals were examined for changes in cell composition (number of macrophages, lymphocytes, neutrophils, eosinophils, epithelial shedding) and mucin levels. At days 3 and 6, there were no differences between the control group (n = 6) and CLN-challenged animals (n = 8) in cell counts and mucin levels. There was no epithelial shedding observed. Substantial increases were seen in the number of eosinophils at day 6 in 1 out of 8 animals in the 100-ng COL-challenged group and in 4 out of 8 animals in the 200-ng COL-challenged group. There was no epithelial shedding and only a minor increase in mucin levels observed in the COL-challenged (200 ng) animals. Based on these results, we concluded that 100 ng per dose of CLN (or COL) does not induce changes that would affect the outcome of allergic airway inflammation induced by RWE or HDME.

Reagents

COL and CLN of bovine origin were obtained from ReGen Therapeutics, Plc., London, England; RWE and HDME was purchased from Greer Laboratories, Inc., Lenoir, N.C., USA.

Statistical Analysis

All data from different mice were analyzed by ANOVA, followed by Fisher's post-hoc analyses for least-significant difference. Data are expressed as the means ± SEM. Results were considered significant at $p < 0.05$.

Results

CLN Does Not Induce, but Decreases RWE- or HDME-Induced IgE Levels

We first tested whether CLN triggers the synthesis of IgE and/or IgG1. Parallel groups of mice (n = 6–11 per group) were sensitized with alum-mixed test material (CNL, COL, HDME, RWE) on days 0 and 4, and on day 11, blood samples were taken to test for IgE and IgG1 levels. Control (naïve) animals received PBS. Results are summarized in figure 1a, which shows that CLN induced an insignificant increase in IgE and IgG1 levels, compared with those seen in unsensitized, PBS-challenged naïve animals. Although COL was reported to have a negligible allergenic potential in humans [27, 28], it induced substantial levels of IgE and IgG1, compared with those in unsensitized groups of mice. HDME and RWE induced high titers of IgE and IgG1, as we have reported previously [21]. Next, we tested whether CLN affects

HDME- and RWE-induced IgE/IgG1 levels. Mice were conditioned with aerosolized CLN (1 mg/ml, twice a day for 3 days) and subsequently sensitized with alum-mixed HDME or RWE. IgE levels were determined 11 days later [24]. Results show that HDME and RWE induced significantly lower IgE levels in CLN-preconditioned mice compared with the levels in mice that did not receive CLN prior to allergen administration (fig. 1b). Treatment of HDME- or RWE-sensitized mice with CLN during allergic sensitization did not significantly decrease the serum levels of IgE specific to HDME or RWE (data not shown).

Lack of Hypersensitivity Skin Test Responses to CLN

Antigen-specific, immediate cutaneous reactivity was examined by skin testing as described previously [26]. Parallel groups of 8-week-old mice were sensitized intraperitoneally with alum-mixed CLN, COL, HDME or RWE, as described in Materials and Methods. Histamine was used as a positive control to induce immediate skin reactions both in naïve and sensitized animals. Two micrograms of test material in a 10-μl volume was injected intradermally (4 per mice), and immediate cutaneous reactions of a wheal diameter ≥3 mm developed within 20 min after injections were recorded. When CLN was administered intradermally to sensitized animals, it resulted in cutaneous reactions that were ≤3 mm (56 out of 56 tests). Higher doses (4 or 8 μg) of CLN also resulted in negative skin test responses. CLN-sensitized animals did not respond to COL, HDME and RWE. Four out of 52 (approximately 7.7%) intradermal COL injections induced moderate skin reactions (3- to 5-mm wheals) in COL-sensitized mice (table 1). On the other hand, positive skin test responses (8- to 12-mm wheals) were observed when intradermal injections of HDME or RWE were given. Among the HDME- or RWE-sensitized mice, approximately 96% (46 out of 48 injection) and about 92% (45 out of 48) demonstrated positive skin tests to HDME ($p < 0.001$) or RWE ($p < 0.001$), respectively. Positive skin test responses were specific to the allergen to which the mice were sensitized. In naïve mice, no cutaneous reactions were observed with CLN, COL, HDME or RWE. Histamine induced wheals of ≥10 mm within 10 min after injection.

CLN Does Not Induce Inflammation and Mucin Production

Six-week-old mice were sensitized intraperitoneally with test articles as described in Materials and Methods. On day 11, parallel groups of sensitized mice (n = 6–9)

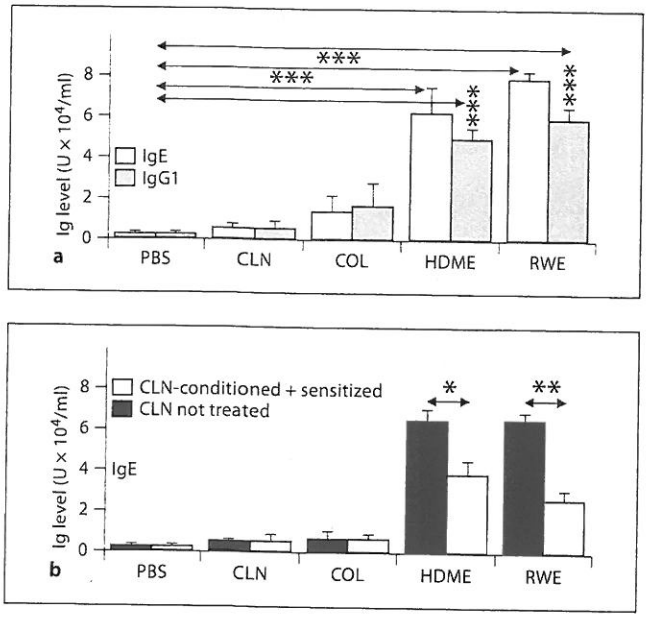
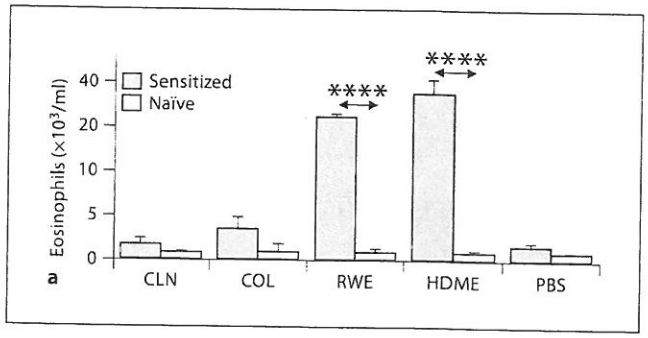


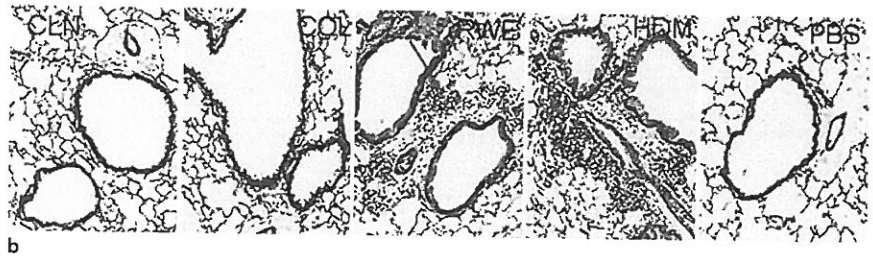
Fig. 1. Effect of CLN on IgE and IgG1 levels. **a** There is no increase in CLN-specific IgE or IgG1 levels in CLN-sensitized mice. Mice were sensitized with alum-mixed CLN, COL, RWE or HDME (150 μg/mouse) on days 0 and 4. At day 11, sera were collected, and levels of IgE and IgG1 were determined by ELISA as in Materials and Methods. **b** Decrease in RWE- and HDME-specific IgE and IgG1 levels in CLN-conditioned mice. Prior to sensitization to RWE or HDME, mice were conditioned with CLN intraperitoneally and sensitized as described in **a**. IgE and IgG1 levels were determined by ELISA as in Materials and Methods. Results are means ± SEM (n = 6–9 mice per group). * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

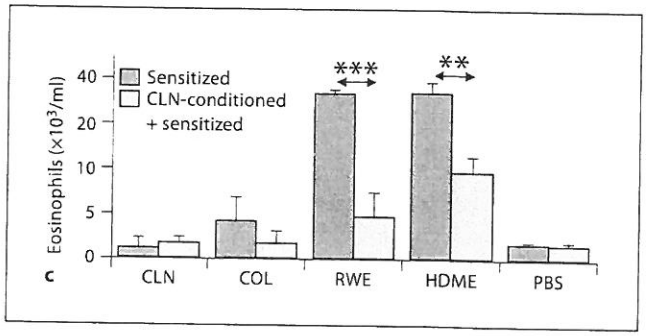
Table 1. Immediate hypersensitivity skin test responses to CLN

Test article	Sensitized	Injection	Wheal diameter mm	Positive reaction	%	p value
CLN	+	56	1–3	0	0	–
	–	32	1–3	0	0	–
COL	+	52	2–5	4	7.7	–
	–	36	2–4	1	2.7	–
HDM	+	48	8–12	46	96	<0.001
	–	28	2–4	1	3.5	–
RWE	+	48	8–12	45	92	<0.001
	–	32	1–4	2	6.2	–
Histamine	+	24	>10	24	100	<0.0001
PBS	+	36	1–3	0	0	–

Eight-week-old mice (n = 8–14) were sensitized with alum-mixed test samples. On day 11, 1 μg of test articles in PBS was injected intradermally. Skin tests were considered positive when the wheal diameter was >4 mm 20 min after substance injection.







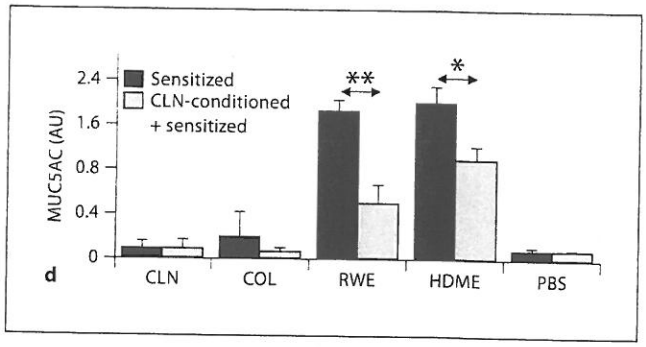


Fig. 2. CLN does not induce inflammation in mice. **a** Low number of eosinophils in BALFs of CLN-challenged animals. Parallel groups of mice were sensitized as described in the legend to figure 1a and challenged intranasally on day 11 with the test article. At 72 h after challenge, BALFs were collected and differential cell counts were done on H&E-stained cytospin preparations. **b** CLN induced no accumulation of eosinophils into the subepithelium. Lung sections were H&E stained. Stained sections were photographed using a Photometrix CoolSNAP Fx CCD digital camera mounted on a Nikon Eclipse TE 200 microscope (magnification ×33). Arrows indicate eosinophils in the subepithelium. **c** Decreased eosinophilia by CLN in RWE- and HDME-challenged mice. Prior to sensitization, mice were conditioned with aerosolized CLN (see Materials and Methods) and sensitized. Sensitized mice were challenged intranasally, and eosinophils migrated into BALF were enumerated at 72 h after challenge of animals. **d** Effect of CLN on mucin levels. Mucin levels in BALF were determined by ELISA as described in Materials and Methods. All data points (**a**, **c**, **d**) are means ± SEM (n = 6–9 mice per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

were challenged intranasally with 20 μg of CLN, COL, RWE or HDME in PBS (80 μl). Control animals received 80 μl of PBS. At 72 h after challenge, mice were euthanized, and BALF was collected to determine the number of inflammatory cells and mucin levels. Lungs were excised as described above for histological examinations. CLN did not induce accumulation of eosinophils either in the BALF (fig. 2a) or in the subepithelium (fig. 2b). In additional studies, delivery of higher CLN concentrations (40 and 80 μg per animal) had no effect on inflammatory parameters (data not shown). Accordingly, differential cell counts showed no significant changes in the number of inflammatory cell types, e.g., macrophages, lymphocytes and neutrophils compared to PBS-chal-

lenged controls. Consistent with a slight increase in IgE/IgG1 levels, mice challenged with COL showed only a small increase in the numbers of inflammatory cells in BALF (fig. 2a). In the subepithelium, there was an inflammatory cell accumulation (fig. 2b). In comparison, the extent of RWE- and HDME-induced inflammation was robust (fig. 2a, b) and similar to that described by our laboratory previously [21]. Naïve mice challenged intranasally with CLN, COL, RWE or HDME showed no markers of inflammation.

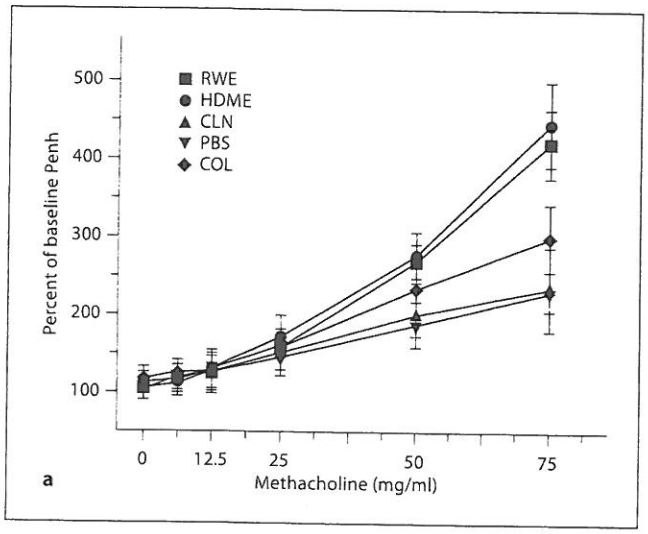
Next, mucin levels of BALFs were evaluated in sensitized and CLN (COL, HDME, RWE and PBS) challenged mice. BALFs were collected at 72 h after challenge, and mucin levels were determined as described in Materials and Methods. Results are summarized in figure 2d. CLN-induced mucin levels are similar to those shown in PBS-challenged animals (<0.1 AU). COL induced mucin secretion into BALF, which was not significantly different from the levels in PBS-challenged controls. After HDME and RWE challenge, mucin levels increased in 9 out of 9 (from <0.1 to 1.8; $p = 0.001$) and 8 out of 8 animals (from <0.1 to 1.7; $p = 0.001$) (fig. 2c).

CLN Decreases RWE- and HDME-Induced Inflammation and Mucin Levels

CLN-conditioned mice produced significantly less IgE against RWE and HDME than did the sensitized, non-conditioned mice, in agreement with the immunomodulatory effects of CLN [2, 3, 6, 7]. Here, we further evaluated the impact of CLN on airway eosinophilia induced by common allergens (RWE, HDME). Parallel groups of mice were conditioned with CLN for 1 week and sensitized with test articles. On day 11, mice were intranasally challenged with CLN, COL, RWE or HDME (20 μg per animal). There was no eosinophilia or increased mucin levels observed in conditioned and CLN-challenged mice (fig. 2c, d). In CLN-conditioned groups of animals, BALF eosinophilia decreased to background levels after COL challenge. When CLN-conditioned mice were challenged with RWE or HDME, we observed a decreased number of eosinophils and mucin levels in BALF, and these were significantly lower than those in non-conditioned groups of animals (fig. 2c, d).

CLN Decreases Airway Response to Methacholine

The airway response to methacholine in mice was assessed by barometric whole-body plethysmography at 72 h after intranasal challenge of sensitized mice with the test article. As shown in figure 3a, the airways of CLN-challenged mice were not significantly more reactive



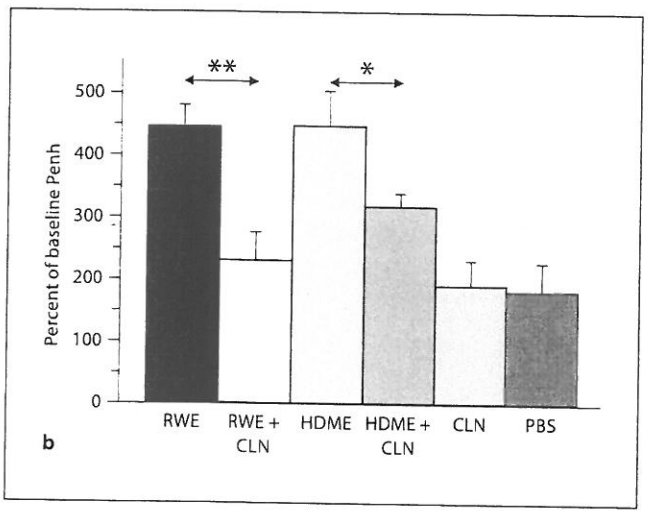


Fig. 3. Effect of CLN on airway responsiveness. **a** There were no significant effects of methacholine on CLN-challenged animals. Animals were sensitized, challenged intranasally, and 72 h later, exposed to increasing concentrations (0, 6.25, 12.5, 50 and 75 mg/ml) of nebulized methacholine. The changes in Penh were recorded by barometric whole-body plethysmography. **b** Decreased Penh in CLN-conditioned RWE- or HDME-challenged animals. Mice were conditioned with alum-free CLN for 1 week and sensitized. At day 11, animals were challenged intranasally with CLN, COL, HDME or RWE, and 72 h later, they were exposed to 75 mg/ml methacholine at 72 h. The changes in Penh were recorded by barometric whole-body plethysmography. **a**, **b** Means ± SEM of Penh values from 3 independent experiments are expressed as the percentage of baseline Penh values observed after PBS exposure. Results are means ± SEM (n = 6–9 mice per group). * $p < 0.01$; ** $p < 0.001$.

than the airways of PBS-challenged controls. For example, the Penh value to 75 mg/ml methacholine was 2.25 ± 0.3-fold for CLN and 2.2 ± 0.2-fold for PBS. In contrast, at 75 mg/ml methacholine, COL-challenged mice showed a substantial increase in airway reactivity compared with PBS-treated animals (2.85 ± 0.3-fold vs. 2.2 ± 0.2-fold; fig. 3a). In HDME- and RWE-challenged mice, the Penh values to 75 mg/ml methacholine were 4.5 ± 0.4- and 4.2 ± 0.3-fold, respectively. When mice were CLN-conditioned and then sensitized/challenged with HDME or RWE, airway responsiveness decreased from 4.5 ± 0.4 to 3.1 ± 0.2 (n = 6) and from 4.4 ± 0.1 to 2.25 ± 0.35 (n = 9), respectively (fig. 3b). These data are consistent with the effect of CLN on HDME- or RWE-induced IgE/IgG1 levels and airway inflammation.

Discussion

CLN is a mixture of low-molecular-weight, proline-rich peptides [2, 4, 5] that has been administered to individuals with age-associated dementia [12–14]. Whether CLN induces allergic responses or modulates responses to other allergens has never been investigated. In the present study, we demonstrate that CLN has no ability to induce IgE antibodies, immediate cutaneous reactions, inflammation, mucin production or airway hyperresponsiveness. COL from which CLN is derived induced low-grade inflammatory responses, which were significantly lower than those induced by RWE and HDME. More importantly, CLN significantly decreased RWE- and HDME-specific serum IgE/IgG1 levels, recruitment of eosinophils and mucin levels in the BALFs of sensitized animals.

IgE is a key player in allergic inflammatory processes, including allergic rhinitis and airway inflammation, asthma, anaphylaxis and allergic gastroenteritis [29]. When IgE antibodies residing on mast cells and basophils come into contact with antigenic epitopes of allergenic molecules, cellular signaling cascades are activated, triggering the release of inflammatory mediators (e.g., histamine, serotonin) and the production of cytokines, such as interleukin (IL)-4 and tumor necrosis factor-α, prior to development of allergic symptoms [30, 31]. Mast cells can also be activated via IgG1 antibodies binding to low-affinity FcγRIII receptors [30, 32]. CLN administration to mice with or without alum resulted in IgE/IgG1 levels that were similar to levels in PBS-treated animals. As shown previously [27, 28], COL induced low levels of IgE/IgG1 antibodies specific to proteins in COL. The lack of an increase in IgE/IgG1 levels after CLN administration is extremely important because these immune globulins are the primary molecules implicated in the initiation of immediate and late-phase allergic responses in sensitized individuals.

Severe IgE-dependent reactions to food, including milk and dietary supplements, are often associated with allergic airway inflammation and respiratory failure, and patients who have asthma are at greatest risk of severe reactions [17–20]. Therefore, we investigated the possibility of IgE-dependent allergic responses to CLN in a well-characterized mouse model [21, 22]. Consistent with an absence of immediate cutaneous inflammatory reactions, intranasal administration of CLN to CLN-sensitized mice resulted in no accumulation of eosinophils in the lungs and/or mucin secretion. Similarly, results from whole-body plethysmography did not show any significant effects of methacholine on CLN-challenged animals. These results support the findings in placebo-controlled studies that reported no side effects or any allergic reactions in CLN-treated healthy volunteers and Alzheimer's disease patients [14].

Taking into consideration the immunomodulatory properties of CLN [3, 7], we investigated whether CLN alters responses to allergenic extracts from house dust mite and ragweed pollen in a mouse model of asthma [21, 24, 33]. The pollen of short ragweed *(A. artemisiifolia)* is one of the most abundant aeroallergens, causing severe seasonal allergic rhinitis, conjunctivitis and airway inflammation in the United States and Europe [34, 35]. HDME isolated from *D. pteronyssinus* and *D. farinae* is among the most common sources of indoor allergens worldwide [36]. More than 50% of patients with allergies and up to 80% of asthmatic children are sensitized to mite allergens [37, 38]. Following CLN conditioning, a decrease in the accumulation of eosinophils and mucin production in the lungs of HDME- and RWE-challenged animals was expected; however, the implicated mechanism requires further investigations. These results are in agreement with data showing that CLN conditioning prior to sensitization with HDME or RWE decreased the levels of specific IgE/IgG1 to these potent allergenic extracts. These findings predict the possible application of CLN to humans for prevention of allergic diseases.

It has been shown that CLN mediates maturation and differentiation of murine thymocytes, promotes proliferation of peripheral blood leukocytes and induces immunomodulators, including various cytokines [2, 3, 6, 7]. Whether or not CLN-induced immune modulators impact processes of allergic sensitization or the manifesta-

tions of allergic reactions are the subjects of speculation. For example, we propose that CLN may alter Th responses and switches chemokine as well as cytokine production towards Th1. Our speculation relies on previously published data showing that CLN alters the production of interferon (IFN)-γ, IFN-α, IL-6 and IL-10 [39, 40]. Th1 cells have been characterized by the production of IL-2 and IFN-γ, whereas Th2 cells secreted IL-4, stimulating IgE production [41]. It is not known if CLN altered IFN-γ levels in our model system; however, it is documented that IFN-γ counteracts IgE production in cell culture systems of both human and mouse origin [42, 43].

It has been shown previously that allergen-bearing pollens induce airway inflammation in sensitized individuals, and the recruited inflammatory cells produce oxidative stress in the airways [44, 45]. We have recently reported that, in addition to their allergenic proteins, ragweed pollen grains or their extracts contain NAD(P)H oxidases, which increase oxidative stress levels in mucosal surfaces and the airway epithelium within minutes of exposure [21, 23]. This oxidative stress is required for antigen-mediated robust inflammation in the lower airways and conjunctiva [21, 23]. In CLN-conditioned mice, the extent of IgE/IgG1 production, cutaneous reactions and airway inflammation was significantly more decreased in RWE-challenged animals than in HDME-exposed mice. CLN-mediated decreases in inflammatory responses to RWE may be explained by the fact that CLN-treated cells have an increased ability to cope with oxidative stress [8, 11, 46]. To a lesser degree, CLN conditioning also decreased HDME-mediated inflammatory responses, although HDME contains proteases to invade the site of exposure and potentiate allergic immune responses [36, 47]. Together, these data suggest that a decrease in RWE- or HDME-induced inflammatory responses by CLN may be explained by an increased capacity of cells for coping with oxidative insults and the immunomodulatory activity of CLN.

In conclusion, these studies show that proline-rich peptides in CLN do not induce allergic immune reactions, including IgE/IgG1 production, immediate cutaneous reactions, late-phase inflammation (e.g., eosinophilia), airway hyperreactivity or mucin production. Following CLN conditioning, a decrease in allergic reactions induced by HDME and RWE was expected; however, the implicated mechanism requires further investigations. We propose that as CLN activates signaling pathways common to the regulation of cell proliferation and differentiation [6, 7, 9], it may also modulate the chemokine and cytokine production of immune cells, thereby altering the response to allergenic antigens. The data described here show that CLN may be used in humans without any concerns with respect to the induction of allergic responses. Further work will be necessary to clarify the molecular basis of our findings and to establish mechanistic correlations between the effects of CLN on immune cells and a decrease in allergic responses to common allergens.

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