Effect of nonfat dry milk and major whey components on interleukin-6 and interleukin-8 production in human intestinal epithelial-like Caco-2 cells

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ABSTRACT

Bovine nonfat dry milk (NDM) and major whey components (lactose, α -lactalbumin, and β -lactoglobulin) were evaluated for their effects on IL-6 and IL-8 production in human intestinal-like Caco-2 cells unstimulated or stimulated with IL-1 β . All the whey components investigated and NDM induced IL-6 production by Caco-2 cells; the most significant increase was observed with β -lactoglobulin. In the case of IL-1 β -stimulated cells, neither NDM nor the major whey components investigated contributed to the induction of IL-6 production after they were stimulated. Induction of IL-8 production by both α -lactalbumin and β -lactoglobulin was higher than that by lactose and NDM; α -lactalbumin was a more potent inducer of IL-8 than β -lactoglobulin and IL-1 β alone in both unstimulated and stimulated cells. In Caco-2 cells that were stimulated with IL-1 β , NDM and all the major whey components investigated had a synergistic effect on induction of IL-8 production, indicating that IL-8 induction was amplified by prior stimulation of cells by IL-1 β . This synergistic effect was not observed with IL-6. Our results suggest that immunomodulatory properties of milk components may be affected by other complex events in the gut.

Key words: immunomodulation, milk, whey, Caco-2 cell

INTRODUCTION

The immunomodulating potential of cow's milk proteins has been receiving much interest because of the growing interest in dairy functional foods and the complex problem of cow's milk allergy. Milk proteins, as well as bioactive milk peptides derived from milk proteins, are thought to have immunomodulating effects (Wong et al., 1998; Clare and Swaisgood, 2000; Swaisgood, 2008) and at the same time cow's milk allergy is the most common type of food allergy in infants (Wal et al., 1995a,b; Wal, 1998; Jarvinen and Chatcha-

tee, 2009). A clear mechanistic understanding on the role of milk and its components in modulating immune function is very much needed.

Whey is a by-product of cheese manufacturing. At one time, it was considered a waste product, but recently it has been elevated to a functional food because of its functionality and its nutritional value. Today, whey is a popular functional ingredient because of its desirable water-holding ability, emulsification, and foaming capacity, which contribute to the quality and organoleptic attributes of numerous food systems (Smith and Culbertson, 2000; Swaisgood, 2008). Whey is also used widely as a dietary supplement promoted to provide antimicrobial activity, immune modulation, and improved muscle strength and body composition, and to prevent cardiovascular disease and osteoporosis (Marshall, 2004). The primary proteins in whey are β -LG and α -LA, representing approximately 50 and 25% of whey proteins, respectively. Whey also contains significant amount of lactose (Swaisgood, 2008)—up to 70% on a dry basis.

The most potent allergen in milk is thought to be β -LG. Approximately 82% of people with milk allergies are sensitive to β -LG (Spies, 1973; Wal et al., 1995b; Adel-Patient et al., 2003).

Although the mechanism as to how milk and its components modulate immune function is not well understood, one possibility is their action through cytokines. Cytokines play an important role in coordinating cell-to-cell communications in immune responses (Goldsby et al., 2000). Of particular interest in gut function are IL-6 and IL-8, which are secreted by intestinal epithelial cells. Their secretion is likely to be regulated by food-derived substances (Satsu et al., 2004). Interleukin-6 is a multifunctional cytokine; it is a B-cell differentiation factor needed for antibody secretion and plays an important role in inflammatory responses (Akira et al., 1993; Goldsby et al., 2000). However, IL-6 also possesses several antiinflammatory characteristics such as downregulation of LPS-induced tumor necrosis factor- α mRNA expression (Miller and McGee, 2002). Interleukin-8 (more accurately a chemokine) functions as a potent chemoattractant and activator of neutrophils and can attract monocytes and T cells to inflammatory

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sites. Interleukin-8 can also have a protective effect, for example, from neutrophil-mediated damage (Gimbrone et al., 1989; Van Damme, 1994; Playfair, 1996; Goldsby et al., 2000). Both IL-6 and IL-8 comprise an important signaling system for the initiation of the mucosal inflammatory response to an antigen. However, cytokine signaling between cells often involves a network of effects. Stimulation of cells by various dietary components before encountering an antigen may amplify the inflammatory immune response to that antigen.

Caco-2 cells have been used extensively as an *in vitro* model for human intestinal epithelial cells because of their physical and functional similarities. They are also good models for immunological studies (Jung et al., 1995; Vitkus et al., 1998; Haller et al., 2000; Hosoi et al., 2003). Caco-2 cells are able to spontaneously differentiate in culture and form tight junctions, and thus resemble normal intestinal epithelial cells. Caco-2 cells also exhibit structures resembling brush border microvilli.

The intent of our research was to determine the role of NDM and major whey components with regard to how they may modulate immune function by their effect on induction of IL-6 and IL-8 production in the Caco-2 intestinal cell model and whether the induction of these 2 key cytokines is amplified by previous stimulation of the cells by the inflammatory cytokine IL-1 β , which is also produced by intestinal cells. We have chosen NDM and the main whey components because these components have been associated with inflammatory immune responses in the past. Our study should provide a better insight to the relative immunomodulatory properties of each of the major whey components and NDM *in vitro*.

MATERIALS AND METHODS

Caco-2 Cell Culture

Caco-2 cells (ATCC HTB-37) were obtained from American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Norcross, GA), 0.01% (vol/vol) antibiotic-antimycotic solution (10.0 U/mL penicillin G sodium, 10.0 mg/mL streptomycin sulfate, and 25.0 mg/mL amphotericin B in 0.85% saline; Gibco), and 0.004% (wt/vol) sodium bicarbonate. Caco-2 cells were first grown in 25-cm² tissue culture flasks at 37°C and 6% CO₂. Cells were loosened from the flask using trypsin-EDTA (Sigma, St. Louis, MO) and harvested by centrifugation at 1,200 $\times$ *g* for 7 min. Cells were transferred to 48-well tissue culture plates (Costar, Cambridge, MA) at 5 $\times$

10⁵ cells/well. Cell numbers were determined using a bright-line hemocytometer (American Optical Co., Buffalo, NY); cell viability was >96%. Monolayers of Caco-2 cells were incubated for 72 h until confluent before use in experiments.

Stimulation of Cytokine Production by Caco-2 Cells

Bovine lactose (Sigma-Aldrich), α -LA (Sigma-Aldrich), β -LG (Sigma-Aldrich) and NDM were suspended in DMEM to obtain a 4% final concentration in cell culture. Interleukin-1 β was used as a positive control because in preliminary experiments, it was observed to be a strong inducer of IL-6 and IL-8, as reported previously by Vitkus et al. (1998). All solutions were filter sterilized using a 0.45- μ m Millex HA syringe filter unit (Millipore Corp., Bedford, MA). First, half (5 out of 10) of the samples were stimulated with IL-1 β (1.0 ng/mL) and the remaining half (5 samples) were left untreated. After 2 h of incubation, solutions containing NDM, lactose, α -LA, and β -LG were added to the monolayer of Caco-2 cells. A negative control was also included. All samples were incubated at 37°C and 6% CO₂ for 24 h. Supernatants were collected and frozen at -80°C until analyzed for the cytokines IL-6 and IL-8 by ELISA. Standards for IL-6 and IL-8 were suspended in DMEM for these experiments. Experiments were replicated 3 times, and the samples were run in triplicate within each assay.

IL-6 and IL-8 Quantification

Procedures included in the OptEIA Set (BD PharMingen, San Diego, CA) were followed for the ELISA. Briefly, 100 μ L of anti-human IL-6 or IL-8 monoclonal antibodies diluted in 0.1 M sodium carbonate buffer (pH 9.5) was added to each well of microtiter strips (Immunolon II Removawell, Dynatech Technologies, Chantilly, VA) set in a Removawell holder (Dynatech Technologies). The plates were incubated overnight at 4°C. Wells were then washed 3 $\times$ with 0.01 M PBS with 0.05% Tween-20 (vol/vol) (PBST) using the Ultrawash Plus ELISA washer (Dynatech Technologies) to remove unbound capture antibody. The plates were then incubated for 1 h with 200 μ L of PBS buffer supplemented with 10% FBS (vol/vol) (pH 7.0) to reduce nonspecific binding. Next, the wells were washed 3 $\times$ with PBST before 100 μ L of standards of recombinant human IL-6 or IL-8 diluted with DMEM with 10% NFDM or sample were added to the wells. Plates were covered with aluminum foil and incubated at room temperature (~24°C) for 2 h. The wells were next washed 5 $\times$ with PBST to remove nonadhering antigens. One hundred milliliters of biotinylated anti-human IL-6 or IL-8 streptavidin-

horseradish peroxidase conjugate (BD Pharmingen) was added to each well and incubated at room temperature ($\sim 24^{\circ}\text{C}$) for 1 h. The wells were then washed 7 $\times$ with PBST before 100 μL of tetramethylbenzidine substrate reagent (BD Pharmingen) was added to the wells. The plates were incubated at room temperature ($\sim 24^{\circ}\text{C}$, 30 min) in the dark. To stop the enzyme reaction, 50 μL of 2 N H_2SO_4 stopping solution was added to each well. Absorbance was read at 450 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). Cytokines were quantified using a standard curve generated from the Softmax curve-fitting program (Molecular Devices). Higher absorbance readings indicated greater amounts of cytokine present in the supernatant sample. All samples were run on one 96-well plate. The intraassay variation for IL-6 and IL-8 was 9.5 and 9.3%, respectively. Cytokine levels in treated Caco-2 cell supernatant were calculated based on the standards and reported in concentration.

Statistical Analysis

Experiments were independently replicated 3 times in a randomized design, and triplicate analysis was run within each assay. Interleukin-6 or IL-8 produced by Caco-2 supernatant and values for treatments were expressed as concentrations.

Data were analyzed by SAS system version 8.2 (2001; SAS Institute Inc., Cary, NC). A value of $P \leq 0.05$ was used as the level of significance. Tukey-Kramer adjustment was used for multiple comparisons.

RESULTS AND DISCUSSION

In this study, production of cytokines IL-6 and IL-8 by Caco-2 cells was studied as a cellular activation marker by NDM and major whey components. This cell line provides a useful model for colon epithelial cells because epithelial cells from fresh colonic tissue are heterogeneous, lack purity, and have limited viability.

Table 1 summarizes the induction of IL-6 and IL-8 production by Caco-2 cells in the presence of NDM, lactose, α -LA, and β -LG unstimulated or stimulated with IL-1 β . All of the major whey components investigated and NDM induced ($P < 0.05$) IL-6 production by Caco-2 cells compared with the untreated controls. However, IL-6 production by NDM (33.0 pg/mL), lactose (35.3 pg/mL), and α -LA (34.4 pg/mL) were similar, whereas β -LG alone induced significantly higher ($P < 0.05$) IL-6 levels (104.3 pg/mL). When Caco-2 cells were stimulated with the proinflammatory cytokine IL-1 β , IL-6 levels in the presence of NDM (58.6 pg/mL), lactose (58.9 pg/mL), and α -LA (62.8 pg/mL) were similar to those in Caco-2 cells stimulated with IL-1 β (67.9 pg/

Table 1. Interleukin-6 and IL-8 production (pg/mL) in human intestinal-like Caco-2 cells treated with NDM and major whey components with or without IL-1 β stimulation

Treatment	IL-6	IL-8
Untreated Caco-2 cells	14.4 $\pm$ 1.4 ^a	179.2 $\pm$ 7.2 ^a
NDM	33.0 $\pm$ 8.2 ^b	177.8 $\pm$ 2.3 ^a
Lactose	35.5 $\pm$ 5.5 ^b	229.4 $\pm$ 66.8 ^a
α -LA	34.4 $\pm$ 1.1 ^b	1,068.4 $\pm$ 101.8 ^b
β -LG	104.3 $\pm$ 4.5 ^c	699.6 $\pm$ 139.6 ^b
IL-1 β	67.9 $\pm$ 1.9 ^d	838.8 $\pm$ 4.7 ^b
IL-1 β + NDM	58.6 $\pm$ 4.2 ^d	1,692.7 $\pm$ 6.1 ^c
IL-1 β + lactose	58.9 $\pm$ 7.4 ^d	1,839.3 $\pm$ 274 ^c
IL-1 β + α -LA	62.6 $\pm$ 7.8 ^d	1,830.7 $\pm$ 152.9 ^c
IL-1 β + β -LG	133.8 $\pm$ 4.0 ^{c,e}	1,763.1 $\pm$ 110.4 ^c

^{a-e}Means within a column with different superscripts are significantly different ($P < 0.05$).

mL) alone. This indicates that these milk components did not contribute further to the induction of IL-6 production in Caco-2 cells after they were stimulated with IL-1 β . Caco-2 cells treated with β -LG and stimulated with IL-1 β showed the highest ($P < 0.05$) level of IL-6 production, but not significantly higher than that achieved in Caco-2 cells treated with β -LG alone. These results suggest that the reported inflammatory effects of β -LG in vivo may be enhanced by IL-1 β in the gut. This is consistent with our earlier observations that Caco-2 cells may require other immune communications before an immune response (our unpublished data).

The effect of NDM and major whey components on induction of IL-8 production did not follow the same trend as that for IL-6 production. Nonfat dry milk and lactose had no effect on IL-8 production in Caco-2 cells. Induction of IL-8 production by both α -LA and β -LG (1,068.4 and 699.6 pg/mL, respectively) was higher ($P < 0.05$) than that by lactose and NDM; α -LA was a more potent inducer of IL-8 production than β -LG and IL-1 β alone in unstimulated and stimulated Caco-2 cell. In Caco-2 cells that were stimulated with IL-1 β , NDM and all of the major whey components investigated had a synergistic effect on induction of IL-8 production, indicating that IL-8 production by the milk component was amplified by the prior stimulation of the cells by IL-1 β .

The primary function of the immune system is the protection of the host from infectious diseases and cancer. However, abnormal responses of the immune system can also lead to disease (e.g., allergies). Desjeux and Heyman (1994) reported that increased protein permeability was not the primary cause of allergies to milk, but rather the allergic response was due to the secondary effect of an abnormal immunological response leading to mucosal inflammation. Very little is known about the effects of milk and its components on the induction of cytokine in intestinal epithelial cells.

Therefore, we chose to look at whole milk components first rather than a digestive model. Both IL-6 and IL-8 are important signaling systems for the initiation of the mucosal inflammatory response to an antigen. Interleukin-6 and IL-8 secreted from intestinal epithelial cells may then affect antibody-forming cells and neutrophils, respectively (Gimbrone et al., 1989; Akira et al., 1993; Van Damme 1994; Playfair 1996; Goldsby et al., 2000). Other peripheral immune cells may also influence cytokine responses of the epithelial cells. Thus, it is important to understand the role of specific food components and how they may be affected by other complex events in the gut to provide a potentially amplified immune response, as we have demonstrated in this study. Our findings certainly do not exclude the possibility that additional cytokines, including other members of the chemokine family, may be involved as a part of this signaling pathway. It is also possible that other cytokines produced by the epithelial cells could amplify other inflammatory responses in the gut. Our results focused on 2 key cytokines and indicate that immunomodulatory effects attributed to milk, particularly whey components α -LA and β -LG, need to be reassessed. Until now, α -LA has received limited attention for its immunological activity. Our results indicate that, under certain circumstances, α -LA can be a more potent immunostimulator than β -LG; further research on α -LA is warranted. Additional studies are also needed to clarify the mechanism of cytokine responsiveness to milk and its components and how this response may be amplified by other important events that occurred prior to or simultaneously in the gut.

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