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Sialic Acid-Containing Milk Proteins Show Differential Immunomodulatory Activities Independent of Sialic Acid

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The immunomodulatory activities of four sialic acid-containing milk proteins (κ-casein, glycomacropeptide, lactoferrin, and proteose peptone-3 component) were determined, and the role of sialic acid was evaluated. Two in vitro models were used: murine splenocyte proliferation, where the effect on LPS-, Con A-, and PHA-stimulated proliferation was studied, and cytokine production in LPS-stimulated murine dendritic cells (DC). All four proteins inhibited LPS-induced splenocyte proliferation, though to different degrees, and independently of sialic acid. κ-Casein strongly inhibited PHA-induced proliferation and had a weak inhibitory effect on Con A-induced proliferation, whereas lactoferrin stimulated Con A-induced proliferation. κ-Casein, glycomacropeptide, and lactoferrin differentially affected cytokine production by DC: κ-casein significantly inhibited production of TNF-α, IL-10, -12, -6, and -1β, independent of sialic acid, whereas less-marked effects of glycomacropeptide and lactoferrin were seen. These findings thus point to important immunosuppressive effects of some milk proteins and indicate that they may function via different mechanisms.

KEYWORDS: Immunosuppression; cell proliferation; dendritic cells; sialic acid; κ-casein; lactoferrin; PP3; glycomacropeptide; LPS

INTRODUCTION

There is increasing evidence that milk not only provides a nutritional supply and passive immunity to the newborn but also contains components with a wide array of biological activities (*1*, *2*). Numerous in vitro as well as in vivo studies have in the past years established that some milk proteins have immunomodulatory properties. Some of these milk proteins are glycoproteins that contain sialic acid (*3*). Sialic acid is a monosaccharide that has been correlated with a range of different biological functions (*4*). It is an important component in milk, as it is of vital importance for growth and development of the newborn (*5*, *6*). Sialic acid is present in milk as components of glycolipids, glycoproteins, and oligosaccharides (*7*, *8*).

Some sialylated glycoproteins, for example κ-casein and lactoferrin (Lf), have been identified as having immunomodulatory activity. κ-Casein is a phosphorylated protein with a molecular weight of approximately 19 kDa (*9*). The glycosylated forms of κ-casein contain 0−5 mol of sialic acid per mol of protein and account for approximately 40% of the total κ-casein molecules (*10*). Previous studies have indicated an immunosuppressive effect of κ-casein (*11*) with the inhibitory activity being attributable to its C-terminal fragment, glycomacropeptide (GMP) (*12*). Lf, an 80 kDa protein containing on average 2 mol of sialic acid per mol of protein (*13*), has been extensively studied, but the results regarding its immunomodulatory properties have, in some cases, been conflicting. Numerous in vitro and in vivo studies identify Lf as an immunosuppressive protein, although immuno-stimulating properties also have been reported (*14*, *15*). To our knowledge, only three studies on the importance of sialic acid for the immunomodulatory activity of GMP have been reported (*12*, *16*, *17*). The role and importance of sialic acid for the activity of κ-casein and Lf, as well as other sialylated milk proteins, still remain to be clarified.

Proteose peptone-3 component (PP3) is another sialic acid-containing protein present in cow's milk. In contrast to Lf and κ-casein, PP3 is not present in human milk. PP3 has a MW of 15.6 kDa and contains on average 0.8 mol of sialic acid per molecule (*18*). There is a growing interest in exploiting biologically active milk proteins in health-promoting products, and the high concentrations of PP3 in bovine milk (300 mg/L) (*19*) therefore represent a readily accessible source. However, there are no previous reports on the immunomodulatory activity of PP3.

The aim of this study was to compare the immunosuppressive effects of the sialic acid-containing proteins, κ-casein, Lf, and PP3, and GMP derived by enzymatic hydrolysis of κ-casein and to clarify the importance of sialic acid in this respect. Two cellular models using either murine splenocytes (proliferation studies) or bone marrow-derived murine dendritic cells (DC)

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(cytokine modulation) were applied. Using these models, we have compared the effect of four milk proteins on the proliferation of spleen cells, a mixed lymphocyte population, stimulated with three different mitogens. Furthermore, using a homogeneous population of DC, we studied the effect of κ-casein, GMP, and Lf on cytokine production induced by lipopolysaccharide (LPS) from Gram-negative bacteria. The activities of neuraminidase-treated κ-casein and Lf were compared to those of native proteins in order to determine if sialic acid is necessary for their activity.

MATERIALS AND METHODS

Materials. κ-Casein was obtained from Sigma (St. Louis, MO). Lactoferrin was purchased from Milei (Stuttgart, Germany). GMP (CGMP-10) was a product of Arla Foods amba (Viby, Denmark). The mitogens lipopolysachharide (LPS, *Escherichia coli* 026:B6), Con A (*Canavalia ensiformis*, concanavalin A), and PHA (*Phaseolus vulgaris*, phytohemagglutinin) were purchased from Sigma.

Preparation of PP3. PP3 was prepared essentially as described in ref 20. In summary, the proteose peptone fraction was prepared from freshly pooled bovine milk, and PP3 was purified from this fraction by Sephadex G-75 gel chromatography, Q-sepharose ion-exchange, and additional Sephadex G-75 chromatography in the presence of urea. For extra high purity, the resulting PP3 was subjected to reverse-phase chromatography on a Vydac C4 column. The purity of the protein was verified by SDS−PAGE and Edman sequence analysis.

Preparation of GMP. A 10 mg/mL solution of κ-casein was prepared in a 10 mM sodium phosphate buffer (pH 6.6) to which recombinant chymosin (0.02 international milk clotting units (IMCU)/mL, 1:1000, w/w) (CHY-MAX Plus, 205 IMCU/mL, 0.92 mg/mL, EC 3.4.23.4, Chr. Hansen AS, Hørsholm, Denmark) was added. After 1 h of incubation at 37 °C (water bath), the pH was adjusted to pH 9.0 with 1.0 M NaOH. After centrifugation (20 min at 12000*g*, room temperature), the precipitate was discarded, and the protein concentration in the supernatant was determined by amino acid analysis (*21*). The supernatant was stored at −20 °C until analysis.

Neuraminidase Treatment. Neuraminidase (from *C. perfringens*, Sigma) was added to a solution of protein in PBS (pH 5.5) (1 mU/μg protein). A control containing no neuraminidase was prepared simultaneously. After 18 h at 37 °C, the reaction was stopped by raising the pH to 7.4 with 1 M NaOH. The sample was stored at −20 °C until analysis.

Determination of Sialic Acid Content. Sialic acid concentration in neuraminidase-treated proteins was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) on a CarboPac PA-01 column (Dionex). A linear gradient with eluents A (0.1 M NaOH) and B (0.1 M NaOH, 1.0 M NaAc) was used. Gradient: 0−10 min (from 93% A, 7% B to 70% A, 30% B); 11−15 min (from 70% A, 30% B to 93% A, 7% B); flow rate 1 mL/min. *N*-Acetyl neuraminic acid (Sigma) was used as a standard.

In Vitro Spleen Cell Proliferation Assay. Single cell suspensions of murine spleen cells from Balb/c mice (7−10 wks) (M&B, Ry, Denmark), fed a standard diet (Altromin 1324, Altromin, Lage, Germany) and water ad libitum, were prepared aseptically by mechanical means and centrifuged for 10 min at 300*g*. Erythrocytes were removed from spleen cell suspensions by treatment with ammonium chloride (8.3 g/l; 5 min on ice) followed by washing two times in Dulbecco's modified eagle medium (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 μg/mL) and streptomycin (100 IU/mL). The cells were finally resuspended in serum-free medium (X-VIVO, BioWhittaker), supplemented with 2 mM L-glutamin, 100 μg penicillin/mL, and 100 IU streptomycin/mL. Cells were cultured as 6×10^5 cells/200 μL per well in quadruplicate in a 96-well flat-bottomed culture plate (Nunc Maxisorp, Roskilde, Denmark) with from 0 (control) to 100 μg of protein/mL/25 μL. Mitogen was added to all wells at a final concentration of 20 μg/mL LPS, 2.5 μg/mL Con A, or 5 μg/mL PHA. Upon incubation at 37 °C in 5% (v/v) CO_2 for 24 h, the cells were pulsed for another 18−20 h with [^{3}H]thymidine (1 μCi/mL; Amersham Biosciences, Buckinghamshire, U.K.) and then harvested onto glass-fiber filter mats using an automatic cell harvester (Autowash 2000, Dynex, Denkendorf). The amount of incorporated [^{3}H]thymidine was determined on a Tri-Carb liquid scintillation analyzer (Packard Instrument (Meriden, CT).

Bone Marrow-Derived DC Culturing. Bone marrow cells were cultured according to the method described in ref 22 with minor modifications. Briefly, femora and tibiae from a female C57BL/6 mouse, 8−12 wk (Charles River Breeding Laboratories, Portage, MI), were removed and stripped of muscles and tendons. The bones were soaked in 70% ethanol for 2 min and rinsed in PBS. Both ends of the bones were cut with scissors, and the marrow was flushed out with PBS using a 27-gauge needle. Cell clusters were dissociated by repeated pipetting. The cell suspension was centrifuged for 10 min at 300*g* and washed once with PBS. Cells were resuspended in RPMI 1640 (BioWhittaker) supplemented with 4 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-ME, 10% (v/v) heat-inactivated fetal calf serum (FCS) (HyClone, Logan, Utah), and 15 ng/mL murine GM-CSF (granulocyte/macrophage colony-stimulating factor). GM-CSF was added as 5−10% culture supernatant harvested from a GM-CSF-producing cell line (*22*). To enrich for DC, 10 mL of cell suspension containing 3×10^6 cells was seeded per Petri dish (day 0) and incubated for 8 days at 37 °C in 5% CO_2. An additional 10 mL of freshly prepared medium was added to each plate on day 3. On day 6, 9 mL from each plate was centrifuged for 5 min at 300*g*, the resultant cell pellet was resuspended in 10 mL of fresh medium, and the suspension was returned to the dish. On day 8, cells were used to evaluate the effect of sialic acid-containing milk proteins on cytokine production as described below.

Induction of Cytokine Production. Nonadherent cells were gently pipetted from Petri dishes containing 8-day old DC-enriched cultures. The collected cells were centrifuged for 5 min at 300*g* and resuspended in medium supplemented with only 10 ng/mL GM-CSF and 1% FCS. Cells were seeded in triplicates in 48-well tissue culture plates (Nunc, Roskilde, Denmark) at a concentration of 1.4×10^6/500 μL/well. Sialic acid-containing proteins were added in three concentrations at 50 μL/well. LPS, (*Escherichia coli* 026:B6, Sigma), at a final concentration of 1 μg/mL, was added. Medium alone was used as a negative control. After a stimulation period of 18−20 h at 37 °C in 5% CO_2, culture supernatants were collected and stored at −80 °C until cytokine analysis.

Cytokine Quantification in Culture Supernatants. TNF-α, IL-1β, IL-6, IL-10, and IL-12(p70) were analyzed using commercially available ELISA kits (R&D Systems Europe Ltd., Abingdon, Oxon, England) according to the manufacturer's instructions.

Statistics. Significant changes in proliferation or cytokine production were analyzed by one-way ANOVA followed by Tukey's post test for multiple comparison. Statistical calculations were performed by GraphPad software, version 3.02 (GraphPad, San Diego, CA). A p value of <0.05 was considered significant.

RESULTS

Effect of Sialic Acid-Containing Proteins on Mitogen-Induced Cell Proliferation. With the use of a cell proliferation assay with murine splenocytes, the effects of four sialic acid-containing milk proteins on proliferation induced by different mitogens (LPS, PHA, and Con A) were compared. Protein concentrations varying from 12.5 to 100 μg/mL were used, which are comparable to the concentrations used in previous studies, thus allowing for comparisons. As shown in **Figure 1**, the proteins differentially affected proliferation depending on the mitogen used.

LPS-induced proliferation was inhibited by all proteins in a dose-dependent manner. Significant inhibition occurred at protein concentrations greater than or equal to 50 μg/mL for GMP, Lf, and PP3, whereas for κ-casein significant inhibition occurred at 12.5 μg/mL. The most pronounced inhibitory effects were seen for κ-casein and Lf (73% and 63% inhibition at 100 μg/mL, respectively) compared to 47% inhibition by GMP and just 18% inhibition by PP3. With regards to κ-casein, inhibition

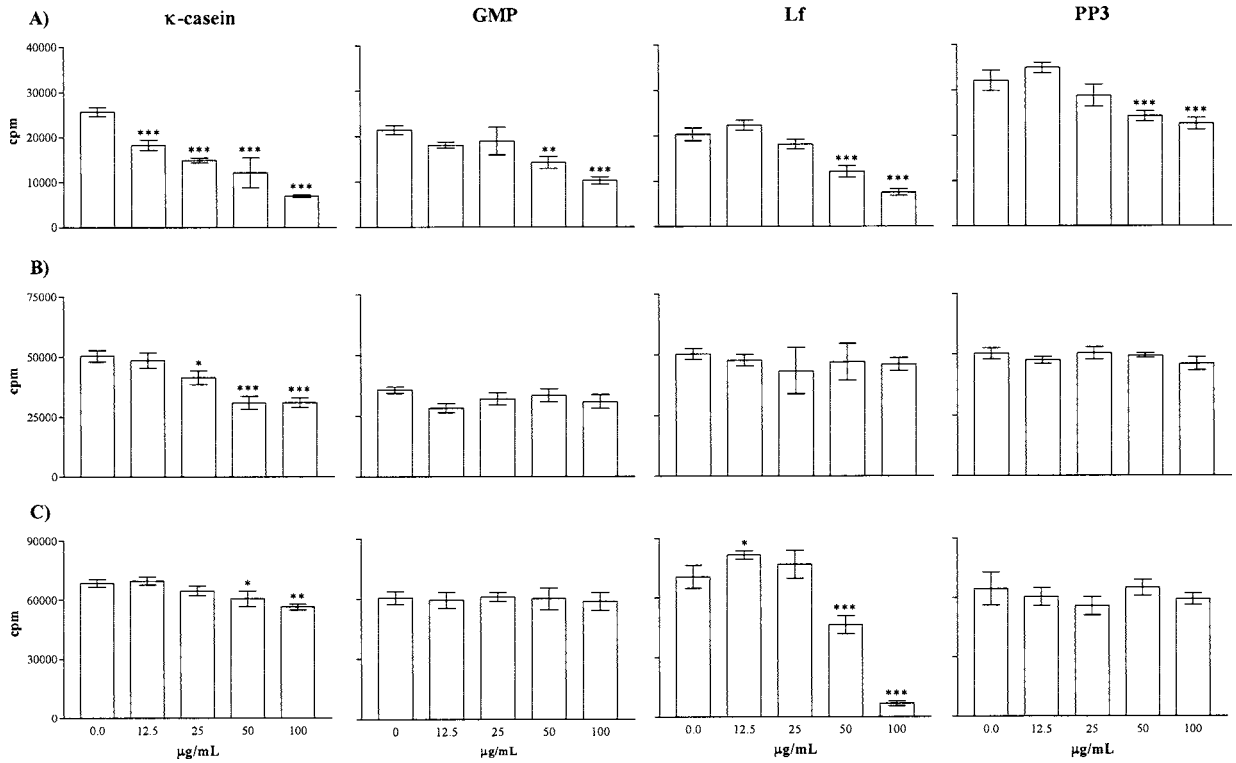

Figure 1. Effect of κ-casein, glycomacropeptide (GMP), lactoferrin (Lf), and proteose-peptone 3 (PP3) on (**A**) LPS-, (**B**) PHA-, and (**C**) Con A-induced proliferation of murine spleen cells. Cells (6×10^5 cells/200 μL/well) were incubated with 20 μg/mL LPS, 5 μg/mL PHA, or 2.5 μg/mL Con A together with 12.5–100 μg/mL κ-casein, Lf, GMP, or PP3. A control without added milk protein was included. Upon incubation at 37 °C in 5% (v/v) CO_2 for 24 h, the cells were pulsed for another 18–20 h with [^{3}H]thymidine. Proliferation was measured as incorporation of [^{3}H]thymidine, and data are presented in counts per min (cpm) and represent the mean ± SD of four wells. Asterisks indicate significant differences between samples with added protein and control samples tested by one-way ANOVA (∗ indicates $p < 0.5$; ∗∗ indicates $p < 0.01$; ∗∗∗ indicates $p < 0.001$). The results are representative of at least three experiments.

of proliferation was observed at concentrations an order of magnitude lower than those for Lf, with significant inhibition occurring at concentrations as low as 5 μg/mL (results not shown). As Lf is approximately 4 times larger than κ-casein, on a molar basis, the inhibitory capacities of κ-casein and Lf are comparable.

PHA-induced proliferation was only affected by κ-casein, resulting in a 38% inhibition at 100 μg/mL.

Lf and κ-casein both affected Con A-induced proliferation, the latter causing an 18% inhibition. Addition of Lf to the cells resulted in a dose-dependent decrease in the incorporation of thymidin, as seen by the fall in cpm. As the lower level of thymidin incorporation may reflect a stimulation so strong that cells have ceased to proliferate at the time of thymidin addition, we tested the dose−response at lower concentrations of Lf. Lf concentrations up to 12.5 μg/mL resulted in an increase in proliferation (66%), where after proliferation decreased, reaching levels lower than the control at concentrations higher than 25 μg/mL (**Figure 2**). None of the other proteins exhibited a stimulatory effect at the lower concentration range (results not shown). Furthermore, it was demonstrated that proliferation was not effected by Lf alone (results not shown).

Whether toxicity could be the cause of the inhibition was ruled out by testing the cell viability upon incubation with protein. No significant changes in cell viability were seen (data not shown).

Effect of Neuraminidase-Treated κ-Casein and Lf on LPS-Induced Cell Proliferation. To clarify the role of sialic acid in the above-described activities, the effects of neuraminidase-treated κ-casein and Lf were compared to the activity of their

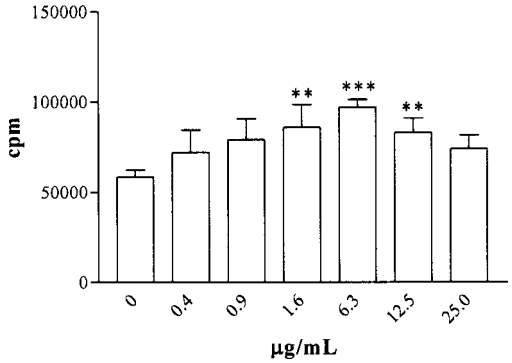

Figure 2. Effect of low concentrations of lactoferrin (Lf) on Con A-induced proliferation of murine spleen cells. Cells (6×10^5 cells/200 μL/well) were incubated with 2.5 μg/mL Con A together with 0.4–25 μg/mL Lf. A control without added protein was included. Upon incubation at 37 °C in 5% (v/v) CO_2 for 24 h, the cells were pulsed for another 18–20 h with [^{3}H]-thymidine. Proliferation was measured as incorporation of [^{3}H]thymidine, and data are presented in counts per min (cpm) and represent the mean ± SD of three wells. Asterisks indicate significant differences between samples with added protein and control samples tested by one-way ANOVA (∗ indicates $p < 0.5$; ∗∗ indicates $p < 0.01$; ∗∗∗ indicates $p < 0.001$).

respective native protein. Removal of sialic acid did not affect the inhibitory activity of either κ-casein or Lf on LPS-induced proliferation (**Figure 3**).

It was controlled that sialic acid was successfully removed by measuring the amount of sialic acid released by neuraminidase treatment using anion exchange chromatography as de-

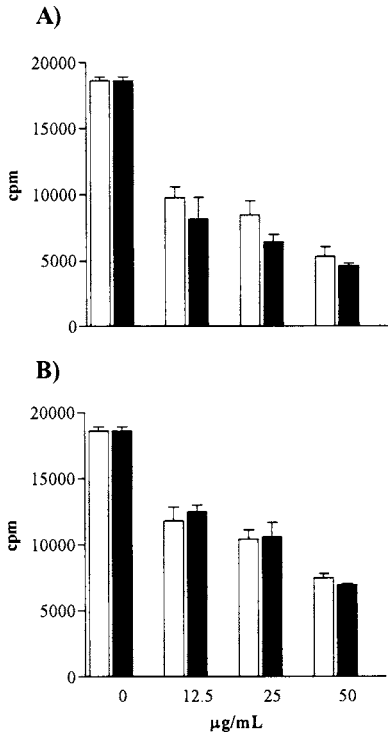

Figure 3. Effect of neuraminidase-treated (**A**) κ-casein and (**B**) lactoferrin (Lf) on LPS-induced proliferation of murine spleen cells. Cells (6×10^5 cells/200 μL/well) were incubated with 20 μg/mL LPS and 12.5–50 μg/mL protein. A control without added protein was included. Upon incubation at 37 °C in 5% (v/v) CO_2 for 24 h, the cells were pulsed for another 18–20 h with [^{3}H]thymidine. Proliferation was measured as incorporation of [^{3}H]thymidine, and data are presented in counts per min (cpm) and represent the mean ± SD of four wells. □: untreated protein. ■: neuraminidase-treated protein. Asterisks indicate significant differences between samples with added protein and control samples tested by one-way ANOVA (∗ indicates $p < 0.05$; ∗∗ indicates $p < 0.01$; ∗∗∗ indicates $p < 0.001$). The results are representative of at least three experiments.

scribed in the Materials and Methods. The values were consistent with the expected sialic acid content of κ-casein and Lf (1.9 mol and 1.6 mol of sialic acid per of mol protein, respectively) (results not shown). Neuraminidase and the enzymatically released sialic acid were not removed from the samples prior to testing them in cell cultures. Therefore, a control containing the enzyme (and without milk protein) was used to ascertain that the enzyme itself did not cause the inhibitory effect observed. A control with free sialic acid was also included. None of the controls had any effect on proliferation (results not shown).

Effect of κ-Casein, GMP, and Lf on LPS-Induced Cytokine Production by DC. The immunomodulatory effects of κ-casein, GMP, and Lf, the proteins that most potently inhibited LPS-induced cell proliferation, were further studied on LPS-stimulated DC. Their effect on LPS-induced maturation of DC was investigated by determining the level of different cytokines produced upon stimulation with and without added protein. κ-Casein showed a significant inhibitory and dose-dependent effect on the production of all five cytokines tested (**Figure 4**). GMP and Lf caused a significant, although weak, reduction in the TNF-α, IL-1β, and IL-6, and IL-12 and IL-1β production, respectively (**Figure 4**).

The effect of neuraminidase-treated κ-casein on LPS-stimulated DC was investigated. As shown for the production of TNF-α, IL-10, and IL-12 in **Figure 5**, the neuraminidase-treated protein exhibited the same inhibitory activity as native κ-casein.

DISCUSSION

The results presented here demonstrate that the four sialic acid-containing milk proteins, κ-casein, GMP (the C-terminal fragment of κ-casein), Lf, and PP3, induce diverse effects on stimulated immune cells with regard to the effect of the individual proteins, as well as their effects toward various stimuli. Sialic acid does not appear to be important for this immunomodulating activity.

Two in vitro cell assays were employed to evaluate the immunomodulatory effects of the proteins: the effects on mitogen-induced proliferation of murine spleen cells and on cytokine production by DC during LPS-induced maturation were studied. In both assays, responses were induced by mitogens in order to enable visualization of immune cell inhibition, as the milk proteins studied are usually observed to inhibit immune responses.

Focusing on the effect of the sialic acid attached to the milk proteins, our study demonstrated that, as removal of sialic acid did not affect the inhibitory activity of κ-casein and Lf on LPS-induced cell proliferation and cytokine production by DC, the activity of the individual proteins was not limited to sialic acid residues. κ-Casein, GMP, and Lf all contain approximately 2 mol of sialic acid per mol of protein. Although similar molar inhibitory potentials of LPS-induced proliferation by κ-casein and Lf (19 kDa and 80 kDa, respectively) were seen, κ-casein and GMP (19 kDa and 7 kDa, respectively) show highly distinct molar inhibitory capacity. Taken together, these results imply that factors other than sialic acid and their localization may determine the activity of the proteins.

As described by Kawakami (*7*), sialylated proteins have been associated with various biological activities, but the role of sialic acid with regards to immunomodulatory activity has, to our knowledge, only previously been studied with respect to the activity of GMP. Otani and co-workers (*12*, *16*) found that removal of sialic acid significantly reduced the inhibitory activity of GMP but concluded that, as neuraminidase-treated GMP was not completely inactive, other factors such as protein sequence may play an import role in determining the activity of GMP. A reduced, but likewise not complete, loss of activity of asialo-GMP compared to that of sialylated GMP was also reported by Li and Mine (*17*). Regarding effects of desialylated proteins, we here tested the activity of desialylated κ-casein, whereas Otani's group (*12*, *16*) used desialylated GMP. However, we find it unlikely that this difference can explain the different results. More likely to explain the discrepancies are methodological differences, such as the mode of neuraminidase inactivation or purification procedures.

The individual milk proteins tested here were found to differentially affect mitogen-induced proliferation of splenocytes, and the effect appeared to depend on the mitogen employed to stimulate proliferation. The mitogens used in this present study are commonly recognized to stimulate different cell populations (*23*, *24*), and hence it seems that the milk proteins affect distinct immune cells. Our results demonstrated that all four proteins inhibited LPS-induced proliferation, the greatest inhibition being by κ-casein and Lf. With regard to the activity of κ-casein, GMP, and Lf, our findings are in accordance with previous reports (*11*, *25*). PP3 was far less potent than κ-casein and Lf.

PHA-induced proliferation was inhibited by κ-casein, a result that is in agreement with previous findings (*26*), whereas no

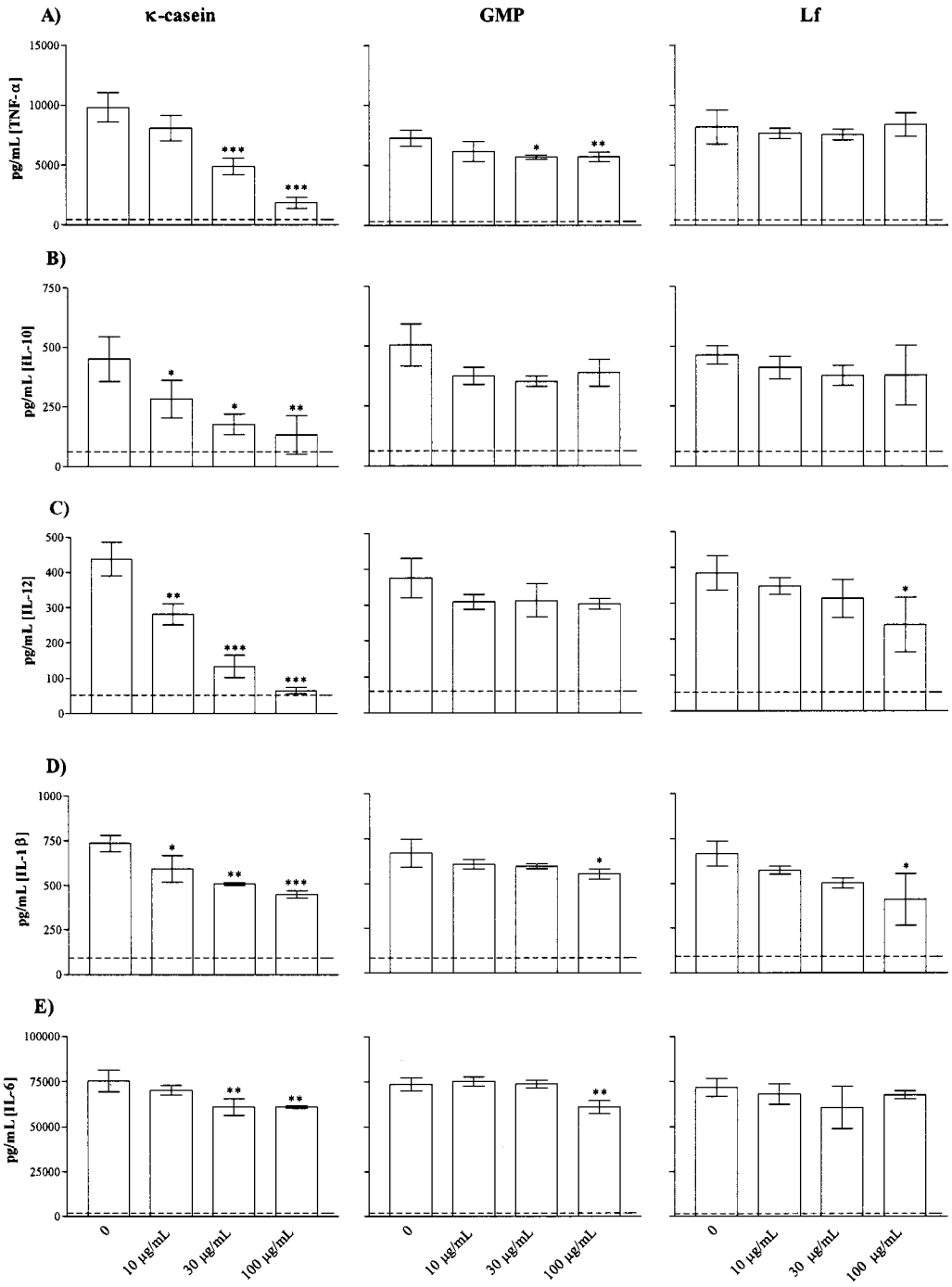

Figure 4. Effect of κ-casein, GMP, and Lf on LPS-induced cytokine production by DC. Cytokines (**A**) TNF-α, (**B**) IL-10, (**C**) IL-12, (**D**) IL-1β, and (**E**) IL-6 were analyzed by ELISA in supernatants collected from 8-day old cultures of murine bone marrow-derived DC cultured for an additional 18–20 h in 48-well plates (1.4×10^6 cells/500 μL/well) with 1 μg/mL LPS and 0, 10, 30, or 100 μg/mL κ-casein, GMP, or Lf. Data represent the mean ± SD values of triplicate wells. The dashed line represents cytokine production in immature DC cultures. The asterisks indicate significant differences between samples with added protein and control samples tested by one-way ANOVA (∗ indicates $p < 0.05$; ∗∗ indicates $p < 0.01$; ∗∗∗ indicates $p < 0.001$).

effect of GMP was seen. In contrast to the results reported by Miyauchi et al. (*25*), we saw no inhibitory effect of Lf on PHA-induced proliferation. However, the degree of iron saturation, which may affect the activity of Lf (*27*), has not been taken into consideration in our study but may explain the discrepancy in the results obtained in the different studies.

Con A-induced proliferation was, in this study, affected by Lf and κ-casein. κ-Casein caused a slight inhibition of proliferation (18%) at 100 μg/mL, which is in accordance to previous findings by Otani and Hata (*26*), whereas Lf stimulated Con A-induced proliferation even at low concentrations (in the interval between 0.9 μg/mL and 25 μg/mL). Con A is a lectin that specifically recognizes mannose and glucose residues in sugar chains (*28*), and as Lf contains two oligomannosidic-type sugar chains (*29*, *30*), it may bind to Con A. We suggest that cross-linking of Con A by Lf may enhance the effect of Con A. In line with our findings, Mincheva-Nilson et al. (*31*) reported a stimulatory effect of Lf on proliferation of human lymphocytes in the presence of Con A.

With the aim of studying how the sialylated milk proteins affect the maturation of DC, a pivotal regulator of antigen specific immune responses, the effect of the milk proteins on LPS-induced maturation of DC was determined by evaluating their effect on cytokine production. The state of activation and

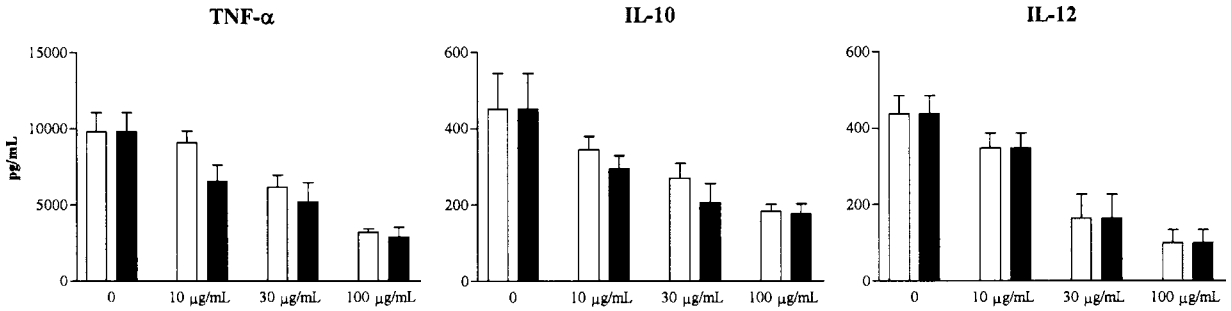

Figure 5. Effect of neuraminidase-treated κ-casein and native κ-casein on LPS-induced production of TNF-α, IL-10, and IL-12 by DC. Cytokines were analyzed by ELISA in supernatants collected from 8-day old cultures of murine bone marrow-derived DC cultured for an additional 18–20 h in 48-well plates (1.4×10^6 cells/500 μL/well) with 1 μg/mL LPS and 0, 10, 30, or 100 μg/mL κ-casein. Data represent the mean ± SD values of triplicate wells. □: untreated κ-casein. ■: neuraminidase-treated κ-casein.

maturation of DC determines their ability to interact with naïve T cells, thereby influencing the type of immune response that is initiated (*32*). These studies may therefore elucidate more about the potential of the proteins to affect immune regulation.

Preliminary studies indicated that PP3 did not significantly affect cytokine production, and we therefore focused on determining the effects of κ-casein, GMP, and Lf. In general, all three proteins exerted inhibitory effects on the cytokine production, although to different degrees. The most prominent effect was exerted by κ-casein, which inhibited all cytokines measured. In contrast, less-pronounced effects of GMP and Lf were seen. There are no previous reports on the effect of milk proteins on cytokine production by DC. With regards to Lf, studies on its effect on LPS-induced cytokine production by human monocytes (*33*−*35*) and mixed lymphocyte populations (*36*) have reported a significant reduction in TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12 production. The less-pronounced effect of Lf seen here may reflect that Lf exerts its effect on specific cell types or may be due to species differences. In line with this, previous studies have demonstrated that Lf appears to act specifically on certain cell types, for example, on Th1 cells but not Th2 cells (*37*), and perhaps via an Lf-specific receptor which has been identified on the surface of both human cell lines and murine lymphocytes (*38*, *39*). Miyauchi et al. (*25*) also suggested that Lf can affect lymphocyte proliferation independently of the presence of macrophage-like cells. Overall, the reduced production of proinflammatory cytokines, especially by κ-casein, points to an antiinflammatory effect of the proteins and support previous findings (*33*, *34*, *36*).

With regard to the mechanisms of inhibition, it has been proposed that the inhibitory effect of Lf is due to its ability to bind to the lipid A moiety of LPS (*40*, *41*), thereby inhibiting subsequent binding of LPS to its receptors. However, as we find that Lf inhibits the production of only selected cytokines in LPS-stimulated DC, as opposed to all cytokines—as would be expected if Lf acted by binding to LPS—it appears that the effect of Lf is by mechanisms other than scavenging LPS. In support of this, Håverson et al. (*33*) reported that Lf affects cytokine production in an LPS-independent manner. In contrast to Lf, κ-casein inhibited the production of all LPS-induced cytokines. This could indicate that κ-casein is cytotoxic or that κ-casein may bind to LPS, thus hindering its action. Cytotoxicity was ruled out as being the cause of inhibition. We also found that κ-casein inhibited proliferation induced by the chemically distinct mitogens PHA and Con A, and we therefore suggest that κ-casein exerts its inhibitory effect by a direct cellular interaction as opposed to an interaction between κ-casein and the mitogen. In line with this, Otani and Monnai have reported that GMP can bind to monocytes (*42*).

Modulation of cytokine production during LPS-induced maturation of DC may influence downstream events in the immune response. Based on the results presented here, we suggest that κ-casein may modulate the activity of DC or other antigen-presenting cells. An inhibitory effect on DC maturation will for example affect T cell activation, production of IL-2, and subsequently proliferation. Such an effect may explain the GMP-induced suppression of IL-2 receptor expression on $CD4^+$ T cells reported by Otani et al. (*43*). The inhibition of IL-1β also correlates well with the finding that GMP increases secretion of an IL-1 receptor antagonist (IL-1ra) (*42*−*44*), both of which point to an immunosuppressive effect of κ -casein/GMP.

Milk has been found to contain different immune modulatory components including gangliosides (*45*) and glycoproteins such as Lf and κ-casein, the presence of which may play important roles in, for example, down-regulating inflammatory responses during immune system maturation**.** Especially κ-casein, which is present in relatively high concentrations in colostrum compared to Lf, and which appears to have a potent inhibitory capacity, may play a pivotal role in modulating immune responses of the intestine upon their early encounters with Gram-negative bacteria.

In this context, the results of the present study add to the understanding of the immune regulatory mechanisms of some milk proteins by comparing the immunosuppressive potencies of four sialylated milk proteins.

In conclusion, the present study compares the immunomodulatory activity of four sialic acid-containing milk proteins. Differential effects are observed, the effect depending on the protein, protein concentration, stimulating mitogen, and cell type studied, which may explain the often conflicting results in the literature. Sialic acid is not important for the activities reported here. The findings in this study suggest an immunosuppressive role for κ-casein, and the reduced production of proinflammatory cytokines point to an antiinflammatory effect.

ABBREVIATIONS

Con A, concanavalin A; DC, dendritic cells; GM-CSF, granulocyte/macrophage colony-stimulating factor; FCS**,** fetal calf serum; GMP, glycomacropeptide; Lf, lactoferrin; LPS, lipopolysaccharide; PHA, phytohemagglutinin; PP3, proteose peptone-3 component.

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