Immunogenicity of κ -Casein and Glycomacropeptide

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ABSTRACT

Glycomacropeptide (GMP), arising from the cleavage of κ -casein by chymosin or pepsin, has been correlated with a wide variety of biological activities including immunosuppression capacity, inhibition of pathogen invasion, and induction of satiety. Due to the interest in exploiting such potential of GMP, we aimed at characterizing the immunogenic properties of GMP as an indication of its potential allergenicity. Immunogenicity of κ -casein and GMP were investigated using 2 animal models based on different routes of immunization: 1) mice immunized intraperitoneally or subcutaneously with either κ -casein, polymerized GMP, GMP coupled to the immunogenic carrier ovalbumin, or GMP alone; 2) mice coadministered κ -casein or GMP and cholera toxin. The specific antibody response to GMP was evaluated as well as the antigen-specific T-cell response. The results demonstrated that immunization or feeding with κ -casein induced GMP-specific antibodies, whereas GMP per se lacked immunogenicity independently of the mode of presentation. The size of the presented form of GMP did not influence its immunogenicity. Because the results showed that GMP did not induce a specific T-cell response, we postulate that GMP lacks the ability to stimulate antigen-specific T cells.

Key words: glycomacropeptide, immunogenicity, immunization route, cholera toxin

INTRODUCTION

Glycomacropeptide (GMP), arising from cleavage of κ -casein by chymosin or pepsin (Eigel et al., 1984), contains no aromatic amino acids and is therefore used for phenylketonuria diets (Smithers et al., 1991). Glycomacropeptide provides good palatability and functional properties imparting favorable mouth-feel and flavor to foods, which many existing food preparations used for phenylketonuria diets lack. Furthermore, GMP has been associated with a variety of biological activities, including binding of cholera toxin and *Escherichia coli*

enterotoxins, inhibition of bacterial and viral adhesions, suppression of gastric secretions, promotion of bifidobacterial growth, and modulation of immune responses (see review by Brody, 2000). It is therefore not surprising that there is a keen and growing interest in exploiting GMP for use in the food industry. The aim of this study was to characterize the immunogenic properties of GMP and κ -casein. Immunogenicity is a prerequisite for the induction of allergy. If a protein is not capable of inducing an immune response by injection with an adjuvant, it cannot be expected to act as an allergen. Accordingly, immunogenicity may be used as an indicator of potential allergenicity.

During various experiments with GMP, we had difficulty in raising GMP-specific antibodies in mice using classical intraperitoneal (i.p.) immunization regimens. Therefore, we speculated that GMP itself might not be immunogenic and that this could be due to its relatively small size (only 7 kDa). The importance of the size of GMP was investigated by immunizing mice with different forms of GMP and determining the antibody response toward GMP.

Others have succeeded in producing anti-GMP antibodies using rabbits immunized intradermally (Collin et al., 1990; Ledoux et al., 1999). We therefore included experiments to study the importance of immunization route.

Because there is an interest in GMP supplementation of foods, it is of relevance to study the influence on the immunogenicity of GMP after oral intake of GMP. However, a common predicament when studying oral immunogenicity of food proteins is the induction of oral tolerance; that is, the active suppression of the systemic immune response to the given food (Strobel and Mowat, 1998). A recently developed murine model, the cholera toxin (CT) model, makes it possible to study the response to orally administered antigens without inducing tolerance. In the CT model, the protein of interest is fed together with CT, the latter serving as mucosal Th-2 skewing adjuvant (Li et al., 1999). We used this model to study the immunogenicity of ingested GMP and κ -casein in mice kept on an essentially milk-free diet, in which the specific antibody and T-cell response were measured.

Received January 31, 2005.

Accepted October 17, 2005.

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MATERIALS AND METHODS

Chemical Modification of GMP

Ovalbumin (OVA; grade 5, Sigma, St. Louis, MO) was covalently coupled to GMP (CGMP-10; Arla Foods aamba, Viby, Denmark) with glutaraldehyde. Solutions of GMP and OVA in 0.1 M sodium phosphate buffer, pH 7.2, were mixed in a 1:1 ratio (wt:wt). Glutaraldehyde (0.4% in phosphate buffer, Merck, Darmstadt, Germany) was slowly added so that the final ratio of mol glutaraldehyde:mol protein was 20:1. A control in which phosphate buffer was added to the mixed proteins was prepared simultaneously. After incubation at room temperature for 2 to 4 h, free binding sites were blocked by adding glycine (in phosphate buffer) to a final ratio of 20:1 (glycine:glutaraldehyde, wt/wt), followed by incubation at room temperature for 30 min. Excess glutaraldehyde and glycine were removed by desalting on a PD-10 column (Sephadex G-25; Amersham Biosciences, Uppsala, Sweden) equilibrated with phosphate buffer. The resulting product was designated **GMP-OVA**.

The procedure for polymerization was the same as described above, adding GMP instead of OVA. The resulting product was designated **pGMP**. All products were characterized by SDS-PAGE.

SDS-PAGE

Sodium dodecyl sulfate-PAGE of GMP products was carried out using precast Tricine porosity gradient gels (10 to 20% monomer, EC6625; Novex, Invitrogen, Groningen, The Netherlands). Samples were mixed 1:1 with sample buffer [0.1 M Tris base, 8% (wt/vol) SDS, 24% (vol/vol) glycerol, 0.025% (wt/vol) Coomassie, 0.04 M, pH 6.8], and boiled for 5 min (Schagger and von Jagow, 1987). Sample lanes were loaded with 10 to 20 µg of protein, or with 2 µL of a molecular weight standard solution (Mark 12, Invitrogen, San Diego, CA). Electrophoresis was carried out for 80 min at constant voltage (125 V). Gels were fixed and stained with Coomassie according to published procedures (Schagger and von Jagow, 1987) but using ethanol instead of methanol.

Immunoblotting

After electrophoresis, the gel was transferred by semidry blotting as described by Plough et al. (1989), with minor modifications. Briefly, the gel was transferred onto a nitrocellulose membrane (0.1-µm pores, 10 × 8 cm, Schleicher and Schuell, Dassel, Germany) for 90 min at room temperature (0.8 mA/cm²) using CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer [10 mM CAPS in 10% (vol/vol) methanol, pH

11.0]. The membrane was blocked for 15 min in Tris-buffered saline with Tween buffer (0.5 M Tris, 0.03 M NaCl, 2% (wt/vol) Tween 20, pH 10.3), and subsequently incubated overnight at room temperature with monoclonal anti-GMP antibody that was produced in our laboratory (0.5 µg/mL) and diluted in Tris-buffered saline with Tween. The membrane was then incubated with alkaline phosphatase-conjugated rabbit anti-mice Ig (1:500, Dako, Glostrup, Denmark) for 1 h at room temperature, and developed for 15 min with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium as substrate.

Immunization of Mice

Groups of female BALB/c mice, 5 to 6 wk old (M&B, Ry, Denmark), were kept in standard animal housing and fed a standard diet (Altromin 1324, Altromin, Lage, Germany) and water ad libitum. The guidelines formulated in "The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" were followed (European Union, 1986). All animal studies were approved by The Danish Animal Experiments Control Body.

Four groups of mice (n = 6) were immunized i.p. with different forms of GMP (10 µg): κ-casein (Sigma), GMP-OVA, pGMP, or a mixture of uncoupled GMP and OVA. Two groups of mice (n = 4) were immunized subcutaneously (**s.c.**) with 50 µg of GMP either in the form of GMP-OVA or as a mixture of uncoupled GMP and OVA. The mice were immunized on d 0 and 14 using freshly prepared protein solutions mixed with Freund's incomplete adjuvant (1:1; 200 µL/mouse, Sigma Chemical Co.). Blood was obtained from the retro-orbital plexus on d 0 and 21 and diluted 1:16 in 0.01 M PBS containing 0.05% Tween (pH 7.4) and stored at -20°C until analysis.

Oral Administration of GMP in the CT Model

Female BALB/c mice, 5 to 6 wk old, were kept in standard animal housing and fed a diet containing very low levels of milk components (Harlan Teklad, Teklad, UK; Brix et al., 2005) and water ad libitum. Two experiments were carried out. The protocol followed the method described by Kroghsbo et al. (2003). Groups of mice (n = 10 to 14) were orally immunized weekly by gavage (200 µL/mouse) for 5 wk with either 1.5 mg of κ-casein or 1.5 mg of GMP together with OVA (2.7 mg), and 10 µg of CT (List Biological Laboratories, Campbell, CA). A group given only OVA served as a control. All animals were deprived of food for 2 h before oral immunizations. The antigen/CT mixtures were freshly prepared in sterile PBS. Blood was obtained from the retro-

orbital plexus on the same day as oral immunization and weekly until 2 wk after the last immunization. Blood samples were diluted 1:10 in PBS-Tween and stored at -20°C until analysis.

Antibody Measurements

For detection of antigen-specific IgG₁, IgG_{2a}, IgM, and total Ig, 96-well microtiter plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with $0.5\text{ }\mu\text{g/mL}$ of GMP in sodium carbonate buffer (0.05 M , pH 9.6). Plates were then incubated with serially diluted blood samples for 1 h at room temperature followed by peroxidase-labeled rabbit antimouse antibodies: anti-Ig ($1.3\text{ }\mu\text{g/mL}$; Dako), anti-IgG₁ ($0.5\text{ }\mu\text{g/mL}$), anti-IgG_{2a} ($0.5\text{ }\mu\text{g/mL}$), or anti-IgM ($0.5\text{ }\mu\text{g/mL}$) antibody (Zymed, San Francisco, CA) for 45 min with agitation at room temperature. Plates were developed with 3,3'-5,5'-tetramethylbenzidine substrate for 10 min in the dark, stopped with 2 M phosphoric acid, and read at 450 nm with 630 nm used as a reference. Titers were determined as the Log_2 to the sample dilution giving an absorbance of 0.2.

Measurement of Antigen-Specific In Vitro Cell Proliferation

Mice were killed by cervical dislocation 1 or 2 wk after the last oral immunization. Single cell suspensions from spleens were prepared aseptically by mechanical means and centrifuged for 10 min at $300 \times g$. Erythrocytes were removed by treatment with ammonium chloride (8.3 g/L ; 5 min on ice) followed by washing twice in Dulbecco's Modified Eagle Medium (BioWhittaker Europe, Verviers, Belgium) supplemented with penicillin ($100\text{ }\mu\text{g/mL}$) and streptomycin (100 IU/mL). The cells were finally resuspended in serum-free medium (X-Vivo, BioWhittaker) supplemented with 2 mM L-glutamine, $100\text{ }\mu\text{g}$ of penicillin/mL, and 100 IU of streptomycin/mL. Cells were cultured at 6×10^5 cells/ $200\text{ }\mu\text{L}$ per well in quadruplicate in a 96-well flat-bottomed culture plate (Nunc) with either $25\text{ }\mu\text{L}$ of PBS (9.5 mM ; BioWhittaker), κ -casein ($25\text{ }\mu\text{g/mL}$), or GMP ($25\text{ }\mu\text{g/mL}$). Upon incubation at 37°C in 5% (vol/vol) CO_2 for 48 h, the cells were pulsed for another 18 h with [^3H]thymidine ($1\text{ }\mu\text{Ci/mL}$; Amersham Biosciences, Buckinghamshire, UK), and then harvested onto glass-fiber filter paper using an automatic cell harvester (Autowash 2000, Dynex, Denkendorf, Germany). The amount of incorporated [^3H]thymidine was determined on a TriCarb Liquid Scintillation analyzer (Packard Instrument, Meriden, CT).

Figure 1. A) Sodium dodecyl sulfate-PAGE of the different glycomacropeptide (GMP) products used for immunization. Lane 1 = molecular mass standards (kDa); 2 = GMP; 3 = polymerized GMP (pGMP); 4 = GMP and ovalbumin (OVA); and lane 5 = covalently coupled GMP + OVA (GMP-OVA). Sodium dodecyl sulfate-PAGE was performed on 10 to 20% tricine gradient gels under reducing conditions and stained with Coomassie; 10 to 20 μg of protein was applied. B) Immunoblot of pGMP (lane 1) and GMP-OVA (lane 2) transferred to a nitrocellulose membrane for 90 min as described in Materials and Methods. The membrane was incubated with monoclonal anti-GMP antibody; alkaline phosphatase-conjugated rabbit antimouse Ig was used for detection.

Statistical Analysis

Significant differences between groups were determined by Student's *t*-test. Statistical calculations were performed by GraphPad software, version 3.02 (GraphPad, San Diego, CA). The *P* value < 0.05 was considered significant.

RESULTS

To study whether the size of GMP could be a determining factor for the immunogenicity of GMP, different GMP products were prepared for immunizing mice. The GMP products were first characterized by SDS-PAGE and immunoblotting (Figure 1). In SDS-PAGE, pGMP (lane 3) is represented by 3 bands with molecular weights corresponding to 17, 31, and 44 kDa, representing 2, 4, and 6 molecules, respectively. Matching protein bands are seen by immunoblotting using monoclonal anti-GMP antibody (Figure 1B). The successful covalent coupling of GMP to OVA (GMP-OVA) is illustrated by the presence of additional protein bands (lane 5). As shown by immunoblotting (Figure 1B), protein bands with molecular weight corresponding to 55, 66, and 77 kDa contain GMP, and represent OVA with 1 to 3 GMP molecules attached, respectively.

Figure 2. Antibody response to glycomacropeptide (GMP) in groups of mice ($n = 6$) immunized intraperitoneally with either polymerized GMP (pGMP), GMP, covalently coupled GMP + OVA (GMP-OVA), or κ -casein. Antibody response was determined by ELISA; titers are calculated as the reciprocal of the dilution giving an absorbance of 0.2 above background, and are illustrated as $\log_2$ -transformed dilutions. Data represent mean titer $\pm$ SD for a group of mice. All titer values for mice immunized with GMP-OVA or κ -casein were significantly greater ($P < 0.001$) than mice immunized with pGMP or GMP (not indicated); * represents a significant difference ($P < 0.05$) between the indicated groups. The dashed line represents the cut-off value ($\log_2$ titer 6.4). The experiment was carried out twice.

Mice were immunized with these GMP products, and the antibody response was characterized. As a control, mice were immunized with a mixture of uncoupled GMP and OVA. Anti-GMP production was induced in mice immunized with GMP-OVA or κ -casein but not in mice immunized with GMP or pGMP, in which no detectable level of antibodies was formed (Figure 2). Characterization of the antibody isotypes revealed that significant levels of IgG₁, IgG_{2a}, and IgM were produced in mice immunized with GMP-OVA or κ -casein. A significantly higher level of IgM was produced in mice immunized with κ -casein compared with those immunized with GMP-OVA.

Regarding the OVA-specific antibody response, there was a significant response only in the groups immunized with uncoupled GMP and OVA, and GMP-OVA (results not shown).

Previous studies demonstrated that anti-GMP antibodies can be produced in rabbits immunized intradermally with GMP (Collin et al., 1990; Ledoux et al., 1999). We therefore investigated whether the route of immunization was of importance for the immunogenicity of GMP. Antibody titers from mice immunized s.c. with either GMP-OVA or a mixture of GMP and OVA

Figure 3. Antibody response to glycomacropeptide (GMP) in groups of mice immunized intraperitoneally (i.p.; $n = 6$) or subcutaneously (s.c.; $n = 4$) with GMP or covalently coupled GMP + OVA (GMP-OVA). Antibody response was determined by ELISA; titers are calculated as the reciprocal of the dilution giving an absorbance of 0.2 above background and are illustrated as $\log_2$ -transformed dilutions. Data represent mean titer $\pm$ SD for a group of mice. All titer values for mice immunized i.p. with GMP-OVA were significantly greater ($P < 0.001$) than mice immunized i.p. with GMP (not indicated); * and ** represent significant differences ($P < 0.05$, $P < 0.01$, respectively) between the indicated groups.

were compared with titer values from i.p. immunizations.

Intraperitoneal immunization with GMP-OVA resulted in significantly greater production of total Ig, IgG₁, and IgG_{2a} compared with s.c. immunization (Figure 3). The level of IgM was not significantly influenced by route of immunization. No significant antibody response was induced in mice immunized with a mixture of GMP and OVA, irrespective of the route of immunization.

Mice were fed (by gavage) either κ -casein or GMP together with CT and OVA and the response toward GMP was determined. There was a significantly greater level of anti-GMP antibody in mice fed κ -casein compared with mice fed GMP. No significant differences were seen for the anti-OVA levels in the 2 groups, confirming the function of the model (Figure 4). Control experiments demonstrated that GMP-specific antibodies were not produced in mice fed only OVA and CT (results not shown).

To study the antigen-specific T-cell response, we tested the in vitro ability of κ -casein and GMP to induce proliferation of splenocytes. κ -Casein significantly induced proliferation in the κ -casein-fed group, but not in the GMP-fed mice. Glycomacropeptide, on the other hand, did not significantly induce proliferation in either the GMP-fed mice or κ -casein-fed mice (Figure 5).

Figure 4. Total Ig antibody response to glycomacropeptide (GMP) and ovalbumin (OVA) in mice fed κ -casein ($n = 13$) or GMP ($n = 9$) together with cholera toxin. Mice were gavaged weekly for 5 wk and blood was drawn weekly. The samples analyzed are after the fifth immunization. Antibody response is determined by ELISA; titers are calculated as the reciprocal of the dilution giving an absorbance of 0.2 above background and are illustrated as $\log_2$ -transformed dilutions. Data represent mean titer for a group of mice ($***P < 0.001$). The experiment was carried out twice.

DISCUSSION

Our results clearly demonstrate that, independently of the route of immunization, GMP alone does not induce the production of GMP-specific antibodies. However, when mice were immunized with κ -casein or GMP

Figure 5. Influence of κ -casein and glycomacropeptide (GMP) on the proliferative response of mice fed κ -casein or GMP together with ovalbumin (OVA), or OVA alone. All mice received cholera toxin. Mice were gavaged weekly for 5 wk; spleens were collected 2 wk later and antigen-specific proliferation was measured for each mouse. Data are represented as the stimulation index (SI) calculated as the response toward a given protein in relation to the background response; data for each mouse represent the mean of 4 wells cultured with antigen ($***P < 0.001$). The experiment was carried out twice.

attached to an immunogenic carrier molecule (OVA), an antibody response toward GMP was generated. Thus, GMP appears not to be immunogenic per se. Substances with a molecular weight less than ~5 kDa are usually inefficient at generating antibody responses, which might seem a likely explanation for the lack of immunogenicity of GMP. However, because pGMP was not able to induce an antibody response, the lack of immunogenicity cannot be attributed to the size of the GMP antigen. Furthermore, as discussed in the literature (Brody, 2000) and as illustrated by SDS-PAGE, GMP has a tendency to aggregate, supporting the notion that size is not the determining factor with regard to immunogenicity of GMP.

Glycomacropeptide per se did not generate an antibody response. However, in mice immunized with κ -casein or GMP-OVA, a significant antibody response was induced. These results suggest that the generation of anti-GMP antibody requires that GMP be coupled to an immunogenic protein, showing GMP to hold haptenic properties.

The higher level of IgM produced after immunization with κ -casein compared with GMP-OVA may be caused by intrinsic properties of κ -casein. Individual κ -casein molecules self-associate via disulfide bonds (Rasmussen et al., 1992) and studies have demonstrated that κ -casein can form micelles (Farrell et al., 1996). Based on the literature as well as our (unpublished) observations, it is likely that κ -casein molecules will orientate with their hydrophobic regions (para- κ -casein segment) forming the core, and their hydrophilic GMP segment projecting outwards (Vreeman et al., 1981). The κ -casein micelle surface, composed of repeated GMP structures, may act as a thymus-independent antigen triggering IgM production in a T-cell-independent manner. In mice immunized with pGMP or a mixture of GMP and OVA, a slightly (but not significantly) elevated level of IgM is induced. This further supports the hypothesis that GMP can only generate GMP-specific IgM antibodies and is not capable of initiating a T-cell-dependent B-cell response.

By comparing the level of antibody production in mice that were immunized either i.p. or s.c., the importance of the route of immunization for the generation of anti-GMP antibodies was evaluated. In agreement with the results from i.p. immunizations, no anti-GMP antibody was detected when mice were immunized s.c. with GMP alone. Thus, the route of immunization did not influence the antibody response in mice immunized with GMP alone. In mice immunized with GMP-OVA, however, those immunized i.p. had significantly greater production of GMP-specific antibodies compared with those immunized s.c. Previous studies reported the successful generation of anti-GMP antibodies after intradermal

immunization of rabbits with GMP (Collin et al., 1990; Ledoux et al., 1999). Subcutaneous immunization involves a site that contains only a few dendritic cells (Bonnotte et al., 2003), which may explain the lower response after s.c. immunization in our study. Dendritic cells are highly efficient antigen-presenting cells and different subsets of dendritic cells exist within different organs (Anjuère et al., 1999). Hence, immunization at different sites may give different responses due to differential antigen recognition by the dendritic cells. The use of different adjuvants (Freund's complete adjuvant vs. Freund's incomplete adjuvant) and the species used for raising antibodies may also influence the response.

Due to the growing interest in exploiting GMP as a dietary component, it is of interest to investigate whether GMP given orally is immunogenic. Using CT as a mucosal adjuvant, groups of mice were coadministered either κ -casein or GMP together with CT. Because previous studies have indicated that GMP can bind to CT (Kawasaki et al., 1992; Oh et al., 2000), mice were also administered OVA. The level of OVA-specific antibodies was determined and used to ensure that interactions between GMP or κ -casein and OVA were not interfering with the model. The level of OVA-specific antibodies was not influenced by the presence of either κ -casein or GMP. Determination of the level of anti-GMP antibody demonstrated that such antibodies could not be detected in blood from mice fed GMP, whereas a significant level of anti-GMP antibody was present when mice were fed κ -casein. As discussed above for the immunization studies, it seems that GMP itself cannot induce generation of anti-GMP antibody whereas κ -casein can. Thus, for ingested κ -casein to generate an anti-GMP antibody response, κ -casein-associated GMP must reach antigen-presenting cells in a nondigested or partially digested form. Studies have demonstrated that κ -casein is readily hydrolyzed by digestive enzymes (Pelissier, 1984; Shammatt et al., 1992). However, only in vitro studies have been reported, which provide no indication of the amount of κ -casein that would resist digestion in vivo. Our study showed that κ -casein, when administered in a concentrated form by gavage, resists digestion to some extent and is absorbed, thus enabling contact with immune cells in the gut-associated lymphoid tissue.

Compared with κ -casein and OVA, GMP has reduced immunogenicity even in the presence of the strong mucosal adjuvant, CT. This suppressed response to ingested GMP could be because GMP is digested during its passage through the gastrointestinal tract although studies have demonstrated that ingested GMP is absorbed and can be detected in the blood (Yvon et al., 1994; Chabance et al., 1998; Ledoux et al., 1999; Fosset et al., 2002). In our own studies on in vitro digestions,

GMP demonstrated a high stability toward pepsin and pancreatin (results not shown).

We also found that GMP was not capable of inducing a T-cell response in either GMP- or κ -casein-fed mice, whereas a T-cell response was induced by κ -casein, although only in cells from κ -casein-fed mice. These results indicate that GMP lacks the ability to stimulate an antigen-specific T-cell response, which may explain the lack of immunogenicity of GMP.

We suggest that GMP is a poor T-cell antigen either because it is resistant to digestion in antigen-presenting cells, or because proteolysis of GMP simply does not give rise to peptides suitable for binding to the major histocompatibility complex.

CONCLUSIONS

The results demonstrated that immunization or feeding with κ -casein gave rise to GMP-specific antibodies, whereas GMP per se lacked immunogenicity independently of the route of presentation. The size of GMP did not appear to be a determining factor for its immunogenicity.

Although GMP may elicit an allergic response in individuals who have already produced anti-GMP IgE (Baldo, 1984), GMP per se does not appear to have the capacity to induce sensitization.

ACKNOWLEDGMENTS

The study was supported by The Danish Program for Advanced Food Technology (FØTEK), The Danish Dairy Research Foundation and Centre for Advanced Food Studies (LMC), Copenhagen, Denmark.

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