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GLYCOMACROPEPTIDE PROTECTS AGAINST EXPERIMENTAL ENDOTOXEMIA AND BACTEREMIA IN MICE

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ABSTRACT

The aim of this study was to evaluate protective effects of glycomacropeptide (GMP), a kappa casein-derived peptide, in experimentally induced endotoxemia or bacteremia in mice. The results showed that BALB/c mice, given intraperitoneally (i.p.) GMP, 24h before intravenous (i.v.) injection of a high dose of lipopolysaccharide (LPS) from *Escherichia coli*, strongly inhibited serum levels of tumor necrosis factor alpha (TNF alpha) and interleukin 6 (IL-6), measured 2h later by bioassays. In addition, GMP, administered 24h before infection of CBA mice with a sublethal dose of *E. coli*, significantly lowered the number of bacterial cells in the spleen. The analysis of main blood cell types in mice pretreated 24h prior to infection with GMP revealed significant increase in the content of granulocytes and immature neutrophils. We, therefore, postulate, that induction of myelopoiesis by GMP may be a primary cause of the increased clearance of bacteria during the development of bacteremia in mice.

Key words: glycomacropeptide, endotoxemia, bacteremia, granulocytes.

INTRODUCTION

Milk is rich in proteins and peptides playing a crucial role in the innate immunity of a newborn and accelerating maturation of its immune system [reviewed in 12, 18].

Glycomacropeptide (GMP), a peptide derived from kappa casein, has attracted considerable interest in the recent years [reviewed in 2]. The peptide is released during the process of cheesemaking by chymosin. The kappa-casein is hydrolyzed into para-kappa-casein (residues 1-105), which remains with the curd, and GMP (residues 106-169), which can be isolated from the whey [20]. GMP has the molecular weight of 8,000 Da and does not contain aromatic aminoacids [22]. GMP possesses a high proportion of sialic acid, which may vary among ruminants [19]. The immunological activities of GMP, such as enhancing effects on phagocytosis and proliferation of human macrophage cell line U937, was particularly increased in GMP rich in sialic acid [10]. Likewise, the inhibitory effect of GMP on binding of cholera toxin to Chinese hamster ovary cells (CHOK1) seemed to be attributed to their terminal sialic acid [6]. In another study, however, both native and desialylated variants of the same bovine GMP totally prevented the adhesion of *Actinomyces viscosus* Ny1, *Staphylococcus sanguis* OMZ9 and *Staphylococcus mutants* OMZ176 to polystyrene surfaces [15]. Still another report [14], regarding effects of several milk-derived proteins on LPS-induced cytokine production, showed no significance of sialic acid in kappa casein in that process but less marked differences in cases of glycomacropeptide and lactoferrin. Taken together, such results suggest that GMP may interact with cells via different types of receptors, i.e. recognizing sialic acid or an aminoacid sequence in GMP molecule. Beside GMP, an inhibitory activity was also exhibited by other mucin-type glycoproteins carrying short O-linked carbohydrate chains. The authors concluded that prevention of adhesion of oral bacteria to polystyrene tubes takes place with no species specificity and can be compared to nonspecific inhibition exhibited by various polymers. In a model of arterial thrombosis, triggered by laser-induced intimal injury in the guinea pig, GMP, the undecapeptide (residues 106-116) and the pentapeptide (residues 112-116) displayed anti-aggregating properties with respect to thrombocytes [1]. That activity was achieved *in vivo* for doses less than one could predict from *in vitro* results. Most promising results derived from studies on infant rhesus monkeys. GMP-enriched formula, given to the monkeys from birth to five months, reduced the degree of diarrhea elicited by administration of enteropathogenic *Escherichia coli* [3]. In a similar model [7] monkeys were brest-fed, fed control, alpha lactalbumin or GMP-enriched formulas. The infants, fed GMP, had higher food intake than did other formula-fed infants. In addition, they had higher plasma zinc and zinc absorption than did breast-fed infants.

Based on our hitherto experience with another milk-derived protein – lactoferrin (LF), which protective roles in the models of experimental endotoxemia and bacteremia were recently demonstrated [11, 9, 23] we wished to investigate potential protective effects of GMP in the respective murine models. Bovine lactoferrin was used in parallel as a reference anti-inflammatory protein. The presented data revealed potent, protective properties of GMP in the studied models. We propose that the ability of GMP to accelerate myelopoiesis may satisfactorily explain the protective action of GMP in bacteremia.

MATERIAL AND METHODS

Mice: CBA and BALB/c mice (12-weeks old) were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy, Wrocław. Mice were fed a commercial, pelleted food and water *ad libitum*. The local ethics committee approved the study.

Reagents: Glycomacropeptide (GMP) was obtained from Tatua Co-Operative Dairy Company, New Zealand. Bovine lactoferrin (LF) (4.4 E.U./mg, <25% iron saturated, was obtained from Morinaga, Japan). The lipopopolysaccharide (LPS) content in the GMP preparation, determined using the method of Rybka and Gamian [17], was 20 nanograms per mg, i.e. 0.002%. LPS (*E. coli* serotype 0111:B4; 3×10^6 E.U./mg) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All tissue culture plates and flasks were purchased from Nunc (Nunc, Denmark).

Treatment of mice with GMP and *E. coli*: GMP or LF were given intravenously (i.v.) to CBA mice in 0.2ml of 0.9% saline into the lateral tail vein, 24h before the *E. coli* strain O55 (10^8 cells suspended in 0.1ml of 0.9% saline). The colony-forming units (CFU) were determined 24h later in the spleens.

Treatment of mice with GMP and LPS: GMP or LF (0.1-10mg doses) were administered i.v. to BALB/c mice in 0.2ml of 0.9% saline into the lateral tail vein, 24h before i.v. injection of 25µg LPS. Control mice were given 0.9% saline only. 2h after LPS injection mice were bled under general anesthesia, serum isolated and kept frozen at -20°C until cytokine determination.

Determination of TNF alpha activity: TNF alpha was determined by the bioassay using WEHI 164.13 cells [4]. Briefly, WEHI 164.13 cells were seeded at a concentration of 2×10^4 cells/well in quadruplicate. Increasing dilutions of the assayed serum were mixed with the target cells in the presence of actinomycin D (1µg/ml). After 20h of incubation, thiazolyl blue (MTT) [5] was added into the wells and the cultures were incubated for additional 4h. Next, a lysing buffer was added and the optical density at 550nm was measured. The detection limit of TNF alpha activity was defined as an inverse of serum dilution where 50% cell death took place.

Determination of IL-6 activity: IL-6 was determined by the bioassay using indicator cell line 7TD1[21]. Briefly, IL-6-dependent murine B cell hybridoma (7TD1 line) was incubated (2×10^4 cells/well) in 96-well plates with serial dilutions of the sera in quadruplicate. Three days later the degree of cell proliferation was determined by the MTT colorimetric method [5]. The detection limit of the assay was about 1.5 pg/ml. One unit of IL-6 activity was defined as an inverse of the supernatant dilution which caused half-maximal proliferation of the indicator cell line 7TD1. The maximal proliferation of the indicator cell line was achieved by application of an optimal concentration of recombinant IL-6.

Determination of colony forming units in the spleen: Bacteria, adjusted to a concentration of 10^8 in 0.2ml of 0.9% saline, were injected into the lateral tail vain of CBA mice. After 24h the mice were sacrificed, the spleens were isolated, homogenized in sterile phosphate-buffered saline (5ml volume), the cell suspension diluted 20× and plated on agar plates in a volume of 0.1 ml. After 24h of incubation the colonies were counted.

Analysis of the cell type profile in the circulating blood: BALB/c mice were subjected to halothane anaesthesia and bled from the retro-orbital plexus. The blood smears were prepared on microscopic glasses, dried and stained with Giemsa and May-Grünwald reagents. The smears were subsequently reviewed histologically.

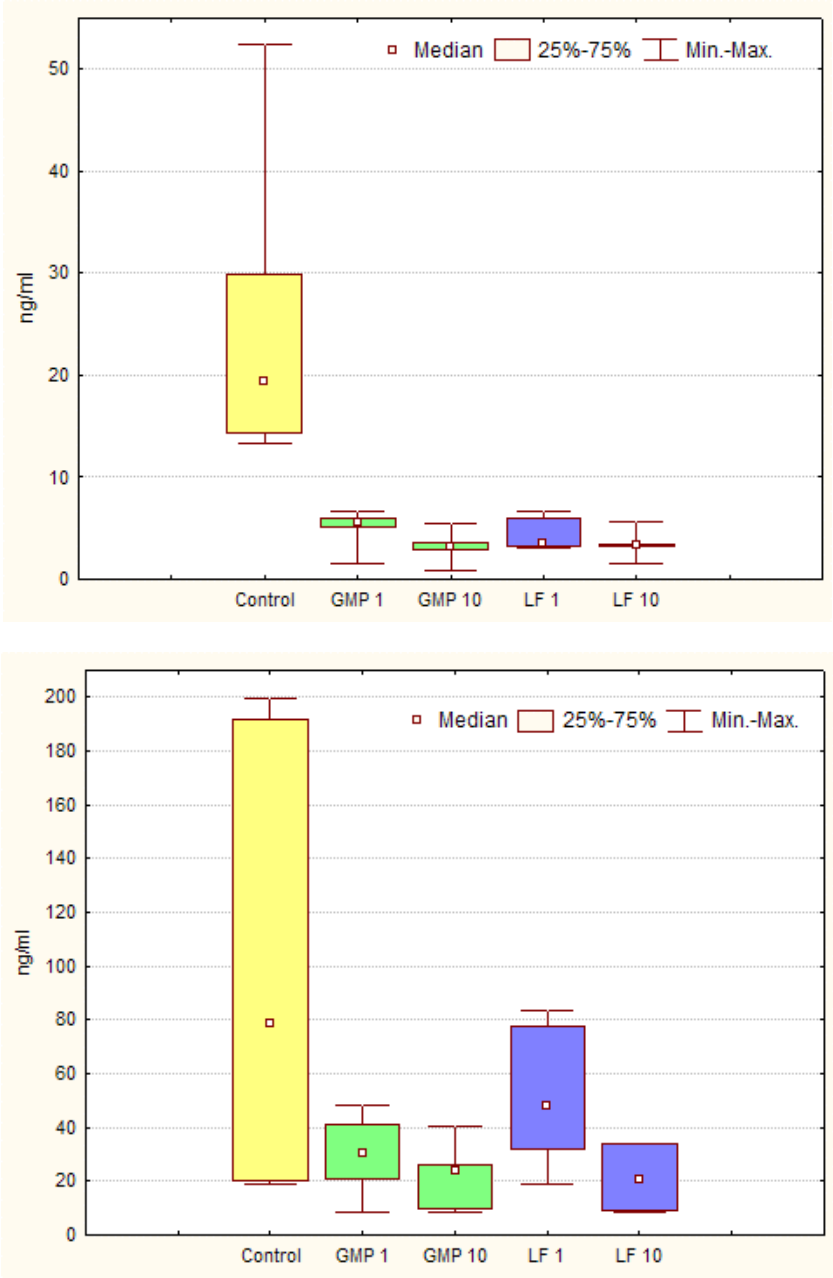
Statistics: The differences were determined by analysis of variance (ANOVA or ANOVA Kruskal-Wallis) after testing homogeneity of variance by Levene's and Brown-Forsyth's test. Individual difference were established using the RIR test (Tukey's test) for multiple comparison. Each experimental group of mice consisted of 6 mice. The results are presented as mean values or median values from 6 determination ±SE. For all tests differences were considered significant when P was less than 0.05. The statistical analysis was performed using STATISTICA 6.0 for Windows.

RESULTS

Effects of GMP on lipopolysaccharide-induced serum levels of pro-inflammatory cytokines

GMP was administered i.v. at doses of 1 and 10mg per mouse, 24h before injection of 25µg of LPS. Lactoferrin, as a reference anti-inflammatory protein, was used at the same doses as GMP. The results (fig. 1AB) showed that pretreatment of mice with GMP led to a strong diminution of the TNF alpha serum level (by 79.4 and 87%, respectively), which was statistically significant for both doses. Similar effects were found with LF. Both GMP and LF caused also a strong reduction of IL-6 levels (69.4 and 77.4% for GMP), although these effects were not statistically significant.

Fig. 1. Effects of GMP pretreatment of mice on LPS-induced serum levels of TNF alpha and IL-6

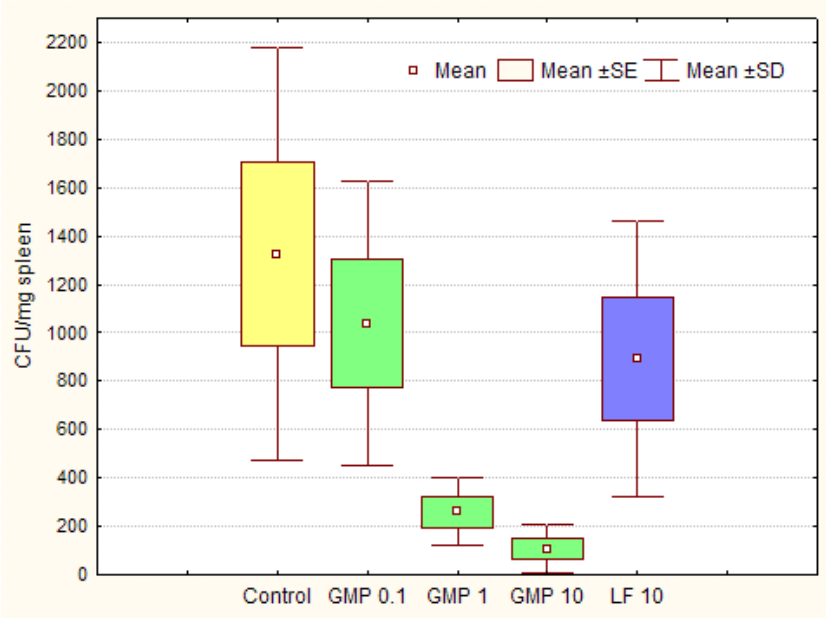


GMP or LF (1 and 10mg doses) were administered i.v. to BALB/c mice 24h prior to i.v. injection of 25µg of LPS. The serum levels of TNF alpha (fig. 1A) and Il-6 (fig.1B) were determined in sera 2h following LPS administration. The differences were determined by analysis of variance (ANOVA Kruskal-Wallis): control/GMP 1 NS; control/GMP 10 $P<0.01$; control/LF 1 NS; control/LF 10 $P<0.01$ (NS – not significant).

Effects of GMP on the clearance of bacteria in the spleens of infected mice

GMP was given i.v. to mice 24h before infection of animals with 10^8 *E. coli*. 24h later the number of CFU in the spleens was determined. The results (fig. 2) demonstrated that GMP significantly lowered the numbers of bacteria in the spleens at 10mg and 1mg doses. The 0.1mg dose of GMP was not effective to lower the number of CFU. Also LF showed no protective effect in this experiment.

Fig. 2. Effects of GMP pretreatment of mice on the number of bacteria in the spleens of infected mice

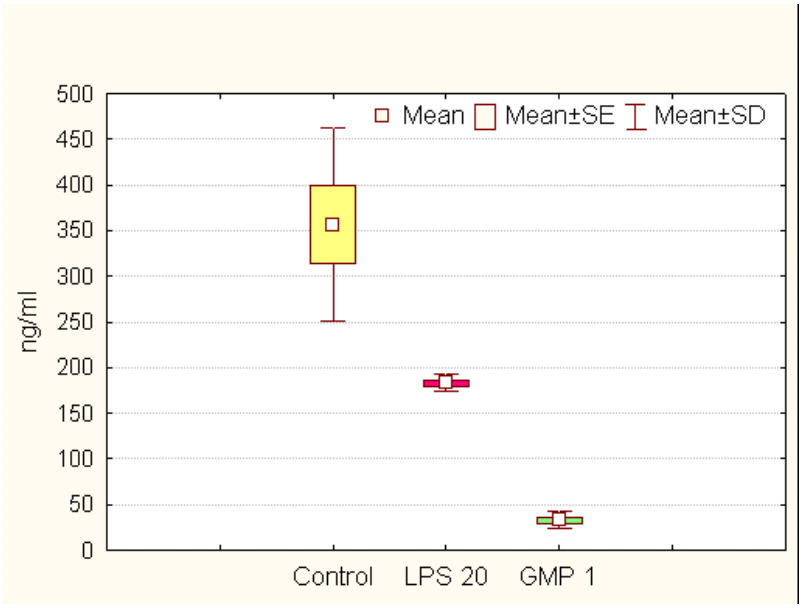


GMP or LF (0.1-10mg doses) were administered i.v. into CBA mice 24h prior to i.v. injection of 10^8 of *E. coli*. 24h later the number of bacterial cells was determined in the spleens. The differences were determined by analysis of variance (ANOVA): control/GMP 0.1 NS; control/GMP 1 P<0.05; control/GMP 10 P<0.02; control/LF 10 NS (NS – not significant).

Evaluation of desensitization of the immune system by equivalent LPS contamination in the GMP preparation

Since GMP, like other biological products, may be contaminated with small amount of endotoxin, which could influence the results, in particular induce desensitization of the immune system, we performed analysis of LPS content by determination of KDO sugars [17]. Here we demonstrate that 1mg of GMP contained 20 nanograms of LPS (0.002%). By application of 20ng of LPS, 24h before injection of the eliciting dose of LPS (25µg) we obtained the following results regarding LPS-induced TNF-alpha serum levels (fig. 3): control (mice pretreated with saline only) – 357ng/ml, 20ng of LPS –183ng/ml and the equivalent dose of GMP (1mg) – 33ng/ml.

Fig. 3. Anti-inflammatory properties of GMP are only partly caused by LPS contamination



BALB/c mice were treated i.v. with 1mg of GMP or 20ng of LPS (an equivalent of LPS contamination). 24h later the mice were injected i.v. with 25µg of LPS and after 2h the level of TNF alpha was determined. The differences were determined by analysis of variance (ANOVA): LPS 20ng/GMP1mg P<0.001; control/LPS 20 P<0.001; control/GMP 1 P<0.001.

Effects of GMP on the content of main blood cell types

Mice were treated with GMP or LF at doses of 1 and 10mg. 24h later the blood was drawn and the content of blood cell types was analyzed. Table 1 shows that the pretreatment of mice with GMP and LF resulted in characteristic alterations in the percentage of cells from the myelocytic lineage. In particular, the content of neutrophil precursors (band forms) was increased following GMP pretreatment. In addition, the percentage of eosinophils was significantly elevated. The increase of mature neutrophil level was also evident. Although increases in the percentage of all granulocytes (effective phagocytes) were not statistically significant for GMP, they ranged, however from 33.5 to 37.6%, which may significantly (see discussion) affect the ability of mice to ingest and remove bacteria from the organs. The changes in the blood picture following LF administration were similar to those of GMP treated mice.

Table 1. Pretreatment of mice with GMP induces myelopoiesis

Dose (mg)	Lymphocytes		Neutrophil precursors		Neutrophils		Eosinophils		All granulocytes (phagocytes)	
	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
Control	75.33	2.70	0.83	0.30	20.50	2.62	3.00	0.44	24.33	2.66
GMP 1	67.16	3.72	2.50	0.56	22.83	2.99	7.16	0.98	32.50	3.82
GMP 10	65.83	3.40	3.66	0.91	23.66	2.37	6.16	1.01	33.50	3.52
LF 1	66.00	2.75	3.66	0.91	24.66	1.96	5.33	0.49	33.66	2.71
LF 10	64.66	2.10	5.16	0.90	24.33	1.85	5.33	0.42	34.83	2.27

BALB/c mice were treated i.v. with GMP or LF (1 and 10mg doses) and 24h later the blood smears were prepared and the analysis of blood cell picture was performed. The differences were determined by analysis of variance (ANOVA): **neutrophil precursors**: control/GMP 1 NS; control/GMP 10 NS; control/LF 1 NS; control/LF 10 $P<0.001$; **neutrophils**: control/GMP 1 NS; control/GMP 10 NS; control/LF 1 NS; control/LF 10 NS **eosinophils**: control/GMP 1 $P<0.01$; control/GMP 10 $P<0.05$; control/LF I NS; control/LF 10 NS; **all granulocytes** (**phagocytes**): control/GMP 1 NS; control/GMP 10 NS; control/LF 1 NS; control/LF 10 NS; **lymphocytes**: control/GMP 1 NS; control/GMP 10 NS; control/LF 1 NS; control/LF 10 NS (NS – not significant).

DISCUSSION

The data presented in this report revealed yet unknown anti-inflammatory properties of GMP. The peptide significantly inhibited the level of TNF alpha in sera of endotoxemic mice and lowered the number of bacteria in *E. coli*-infected mice. Although the mechanism of desensitization of mice to LPS can not be at present satisfactorily explained, the action of GMP on the clearing of bacteria could indeed be a consequence of the significant increase in the content of phagocytes, as shown in table 1. In fact, others demonstrated that a 60% increase of neutrophil content may stimulate the clearing process of bacteria by 6.5 fold [8]. Such a mechanism of protection against bacteria was also confirmed for LF [23]. Since GMP is a derivative of casein we suppose that the mechanism of GMP action in the described model may be similar to that found in so-called “sterile inflammation” phenomenon, induced by a subcutaneous injection of casein [23], and mediated by liberation of granulocyte colony-stimulating factor.

The results shown in figure 3 indicate that although the equivalent dose of LPS caused some desensitization of the immune system, as evidenced by a marked decrease of the TNF alpha serum levels, the GMP molecule has to contain certain immunotropic epitopes, responsible for a much more profound inhibitory effect, statistically significant in comparison to LPS control group. A prime candidate for such an epitope could be GMP sugar moiety, rich in sialic acid, which could interact with sialoadhesin receptor on macrophages [13], although not all reports are unequivocal in this respect. It seems also unlikely that the protective effect of GMP in endotoxemia, could be due to neutralization of LPS, since the peptide was administered 24h before injection of LPS. More likely, the mechanism of the protective effect of GMP in endotoxemia may also be associated with its ability to induce production of pro-inflammatory cytokines in target cells, which in turn may lead to a transient hyporeactivity. In our pilot experiment we found that GMP at a very low dose (0.1µg/ml) increased 2-fold the spontaneous production of TNF alpha and 8-fold the production of IL-6 in the cultures of peritoneal rat cells (data not shown). The stimulation of TNF alpha and IL-6 production by 1µg/ml of GMP was 8-fold and 16-fold, respectively. On the other hand, GMP had no influence on LPS-induced cytokine production in that model, which again excludes a possibility of LPS inactivation by forming LPS:GMP complexes or prevention of LPS interaction with LPS cellular receptor.

Finally we demonstrated that LF, a well described anti-inflammatory molecule, was less potent in protection against development of endotoxemia and bacteremia than GMP.

In summary, this study demonstrated a potential utility of GMP in prophylaxis of certain type of infections. Desensitization of the immune system and stimulation of myelopoiesis seems to be key factors for the protective effects of GMP. These results represent a contribution to the present knowledge on medical benefits of milk-derived proteins and peptides.

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