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Short communication

Evidence for correlation between the intensities of adjuvant effects and NOD2 activation by monomeric, dimeric and lipophylic derivatives of *N*-acetylglucosaminyl-*N*-acetylmuramyl peptides

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Abstract

Adjuvanticity of a series of peptidoglycan fragments—known as muramyl peptides—and their lipophylic derivatives was examined and compared with the ability of these compounds to activate NF–kB pathway through NOD2.

The adjuvant activity of di, tetrasaccharide peptides and stearoyl containing derivatives has at least two peaks in dose–response curves and the greater of them correlates with respective dose–response data for NF–kB stimulation through NOD2. Introduction of stearoyl moiety, with the aim of improving muramyl peptide interaction with the cell membrane and subsequent intracellular delivery, influenced the corresponding activities in vitro, but did not correlate with improved effects in vivo experiments.

IgG subtypes tests indicate that muramyl peptides preferentially stimulate IgG_1 production, whereas the tetrasaccharide containing muramyl peptide additionally induces production of IgG_{2b} subclasses.

On the whole, comparison of the adjuvanticity in vivo and the NOD2 activation in vitro revealed a clear correlation between the two responses. These findings confirm the view that NOD2 pathway activation should account, at least in part, for the adjuvant effects of these compounds.

Keywords: Muramyl peptides; Adjuvant; NOD2

1. Introduction

The first activity that has been established for muramyl peptides, which are the minimal structures of bacterial cell wall, was adjuvanticity [1–3]. However, the molecular mechanism underlying this effect remained unclear. Recent studies [4,5] led to the discovery of intracellular receptors for peptidoglycan components, namely NOD1 and NOD2, which belong to a family of proteins named nucleotide-binding site/leucine-rich repeat protein (NBS/LRR). S.E. Girardin et al. [6] reported that NOD1 recognizes unique diaminopimelate (DAP)-containing muramyl peptides whereas NOD2 interacts with the muramyl dipeptide [7,8]. NOD2 is expressed mainly in macrophages and other cells of myeloid lineage, which are considered as dominant target cells responsible for the adjuvant activity of muramyl peptides.

In this study we looked for a correlation between the stimulation of the secondary antibody response to a pro-

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tein antigen by series of muramyl peptides in vivo with the NF–kB activation via NOD2 by the same compounds in vitro.

2. Materials and methods

2.1. Peptides

N-acetyl-D-glucosaminyl-(β1 → 4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (GMDP), *N*-acetyl-D-glucosaminyl-(β1 → 4)-*N*-acetylmuramyl-L-alanyl-D-glutamic acid (GMDPA), *N*-acetyl-D-glucosaminyl-(β1 → 4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine-L-lysine (GMDP-Lys), (*N*-acetyl-D-glucosaminyl-(β1 → 4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine)$_2$ (GMDP)$_2$, (*N*-acetyl-D-glucosaminyl-(β1 → 4)-*N*-acetylmuramyl-L-alanyl-D-glutamic acid)$_2$ (GMDPA)$_2$, (*N*-acetyl-D-glucosaminyl-(β1 → 4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine-L-lysine)$_2$ (GMDP-Lys)$_2$ were synthesized in the title institute by methods described in Refs. [3,9–11]. *N*-acetyl-D-glucosaminyl-(β1 → 4)—*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-ε-stearoyl-L-lysine (GMDP-Lys(St)) and *N*-acetyl-D-glucosaminyl-(β1 → 4)-*N*-acetylmuramyl-L-alanyl-D-glutamyl-ε-stearoyl-L-lysine (GMDPA-Lys(St)) were prepared by acylation of the ε-amino moiety of GMDP-Lys and GMDPA-Lys (in turn prepared as in Ref. [11]) by *N*-hydroxysuccinimide ester of stearic acid in *N*,*N*-dimethylformamide in 45–50% yield. The structures of all synthesized compounds were confirmed by ^{1}H NMR spectroscopy and MS; the peptide purity was determined by HPLC and was not less than 97–98%. LAL test [12] of all substances gave negative response. Peptide solutions were sterilized before using by filtration through pyrogen-free filters (FlowPoreD pore size 0.2 μm).

2.2. Animals

BALB/c, female 8-week-old mice were obtained from the Central Animals Facility (Moscow) and housed under conventional condition.

2.3. Antibody response

Mice, 8–10 animals per group, were immunized with ovalbumin (25 μg/animal) simultaneously with different doses of glycopeptides by intraperitoneal injections. Two and four weeks later, mice were boosted with ovalbumin (12.5 μg/animal) and one week after the last boost serum samples were obtained and tested by enzyme-linked immunosorbent assay (ELISA).

2.4. ELISA

Ninety six-well plates (Nunc) were coated with ovalbumin (500 ng/well) overnight at 4 °C. Plates were washed and saturated with 1% bovine serum albumin (BSA) in PBS at 37 °C for 1 h. After washing serial dilutions of pulled antiserum, obtained from each animal group, were added in triplicate for overnight at 4 °C. Plates then were washed and goat anti-mouse IgG (H + L) peroxidase conjugate (BioRad) was added at 1:3000 dilution. Plates were washed and the solution of *o*-phenylenediamine (OPD) in 1% citric acid (pH 4.4), plus 0.01% H_2O_2 were added for 10 min at 37 °C. Reaction was stopped by 2N sulfuric acid. Titers were expressed as $\log_2$ at $OD_{492} = 1.0$ and stimulation index was estimated as ratio of antiserum titer obtained in the presence of tested glycopeptide to antiserum titer obtained without any adjuvant. Data presented in Section 3 are an average of at least three independent experiments.

Goat anti-mouse IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 peroxidase conjugates (Southern Biotechnology Associates) were used for measuring the titers of IgG subclasses; the titers are expressed as $\log_2$ at $OD_{492} = 0.3$.

2.5. Cells and reagents

Human embryonic kidney HEK293T epithelial cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Prior to transfection, HEK293T cells were seeded into 24-well plates at 10^5 cells/ml density as described in Ref. [13].

2.6. Expression plasmid and transient transfection

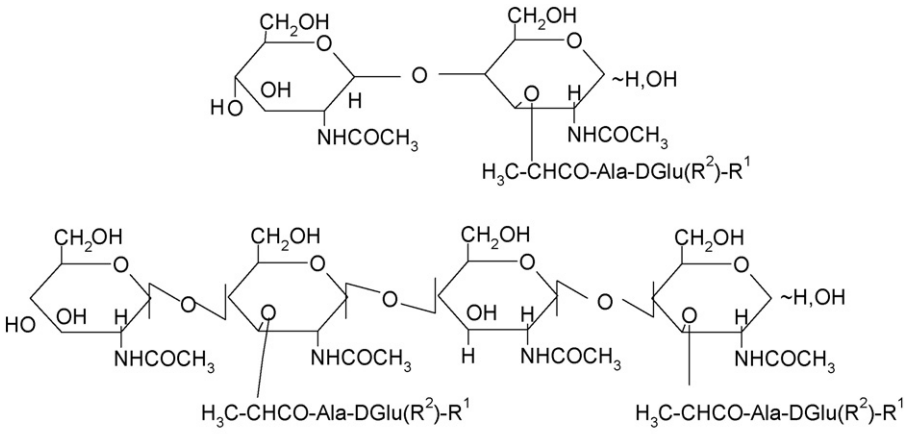
The expression plasmid for NOD2 was from Gilles Thomas (Foundation Jean Dausset/CEPH, Paris, France). Transfections were carried out in HEK293T cells as described in Ref. [13].

2.7. NF–kB activation assay

Studies examining the synergistic activation of NF–kB by muramyl peptides in cells over-expressing NOD2 were carried out as described in Ref. [14]. Briefly, HEK293T cells were transfected overnight with 30 ng of NOD2 plus 75 ng of Igk luciferase reporter plasmid. At the same time, different amounts (10, 50 or 250 pmol) of muramyl peptides were added to the cell culture medium, and the synergistic NF–kB-depended luciferase activation was then measured following 24 h of co-incubation. NF–kB-depended luciferase assay were performed in duplicate, and the data shown in Section 3 represent an average of at least three independent experiments.

2.8. Statistics

Statistical significance ($P < 0.05$) of antibody production experiments was determined by Student's *t*-test. Data of NF–kB activation assay show mean ± S.E.



	R^1	R^2
1. GMDP	NH_2	OH
2. GMDPA	OH	OH
3. GMDP-Lys	NH_2	-Lys
4.$(GMDP)_2$	NH_2	OH
5.$(GMDPA)_2$	OH	OH
6.$(GMDP\text{-}Lys)_2$	NH_2	-Lys
7. GMDP-Lys(St)	NH_2	-Lys(St)
8.GMDPA-Lys(St)	OH	-Lys(St)

Fig. 1. Structure of glycopeptides.

3. Results

3.1. Adjuvant activity

Structure and names of tested compounds are shown in Fig. 1. We divided all investigated glycopeptides in three groups: di, tetrasaccharide and stearoyl containing derivatives. Adjuvant activity of muramyl peptides was estimated by their stimulatory effect on secondary humoral response to ovalbumin in mice. Only the first immunization procedure included muramyl peptide injection simultaneously with ovalbumin while the two following boosts were done only with the protein. Data of antibody production assay by ELISA are shown in Fig. 2A–C. In each peptide group, at least one peptide was examined in a broad concentration range. In those cases, the main and the minor peaks were present in dose–response dependencies (Fig. 2A compound 1; Fig. 2B compounds 2 and 3; Fig. 2C compound 1). From peptides of the first group, GMDPA showed the highest adjuvant activity at three high doses ($P = 0.02$), but at the fourth (lower) dose, GMDP-Lys was the most active ($P < 0.01$).

A – "dimer" – of GMDP-Lys was the most active adjuvant among the second group at two highest doses ($P < 0.05$). A – "dimer" – of GMDP was next active while a – "dimer" – of GMDPA demonstrated the lowest activity among the peptides of the second group. It should be noted that "dimers" of peptides with the exception of $(GMDPA)_2$ are the most powerful adjuvants among all tested peptides.

In the third group GMDP-Lys(St) showed maximal activity at lower peptide doses compared to the peptides of the first two groups ($P = 0.05$). At higher doses, however, the stimulatory effects were only moderate. On the contrary, GMDPA-Lys(St) induced considerable stimulation even at moderate dose.

To estimate the distribution of IgG subclasses stimulated by muramyl peptides, we have chosen, for assay, three compounds from each peptide group and tested three antisera corresponding to the maximum value of stimulation index: GMDP at 144 nm; $(GMDP\text{-}Lys)_2$ at 7.2 μm and GMDP-Lys(St) at 14.4 nm. In all three cases, as well as in the case of the control group (titer 4.0 ± 0.1), primarily the IgG_1 subclass was present and stimulation index for all tested compounds was around 4.5. Interestingly, $(GMDP\text{-}Lys)_2$ also elicited production of IgG_{2b} with stimulation index 2.8 (the titer of the control group was 1.5 ± 0.1).

3.2. NOD2 activation

To examine the influence of muramyl peptide structure on NOD2 sensing, we tested NF–kB activation using a luciferase reporter assay in the human embryonic kidney epithelial cell line, HEK293T, over-expressing NOD2

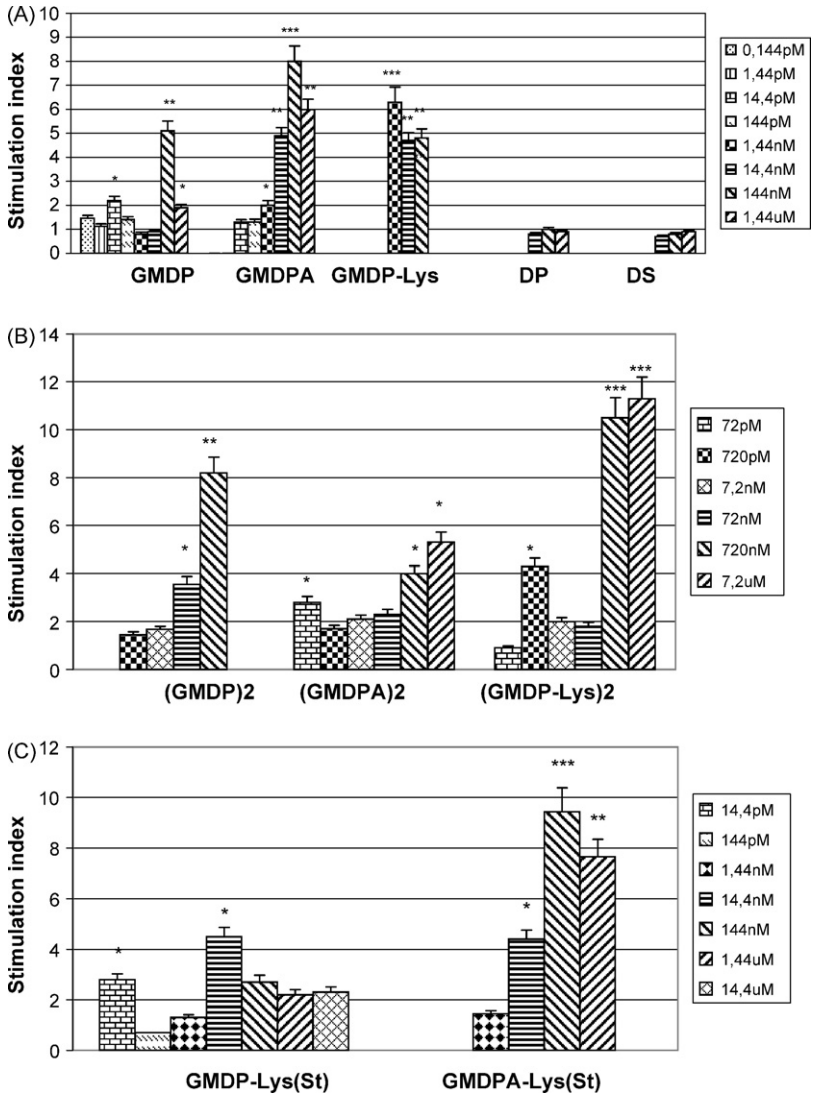


Fig. 2. Secondary antibody response to ovalbumin in mice, stimulated by: (A) disaccharide glycopeptides, DS-disaccharide, DP-dipeptide; (B) tetrasaccharide glycopeptides and (C) disaccharide glycopeptides with ε-stearoyl-lysine. In diagram, legends peptide doses were indicated per animal. Serum titer was estimated by ELISA and the stimulation index are presented as ratio of antiserum titer, obtained with glycopeptide as adjuvant to antiserum titer, obtained without any adjuvant. $^{***}P<0.001$, $^{**}P<0.03$, $^{*}P<0.05$ vs. control.

in the presence of a range of peptide doses. These data are shown in Fig. 3A–C. All tested compounds were indeed NOD2 agonists. In the first group (Fig. 3A) at maximal peptide doses, the NOD2-dependent NF–kB activity was highest for GMDPA and lowest for GMDP (GMDPA > GMDP-Lys ($P=0.05$) > GMDP ($P<0.05$)). At lower dose, however, GMDP appeared to be more active than GMDPA (i.e., GMDP > GMDPA > GMDP-Lys) ($P=0.01$). In the second group (Fig. 3B) $(GMDP\text{-}Lys)_2$ had the greatest stimulatory effect at the high doses: $(GMDP\text{-}Lys)_2 > (GMDP)_2$ ($P<0.001$) $\geq (GMDPA)_2$. At low doses $(GMDP)_2$ appears more active at stimulating NOD2 (i.e., $(GMDP)_2 > (GMDP\text{-}Lys)_2$ ($P<0.001$) $\geq (GMDP)_2$). Both compounds from the third group, i.e., stearoyl derivatives, expressed relatively high activity at all tested concentrations.

4. Discussion

Comparison of dose–response dependencies obtained for different muramyl peptides in terms of their ability to activate NF–kB via NOD2 in vitro and their adjuvant activity in vivo revealed similar trends in several cases. For example, among the disaccharide peptides, GMDPA showed the strongest effects in both tests at high doses, while at lower doses, GMDP became the most active adjuvant and also NOD2 agonist. In the case of GMDP-Lys, the steep dose–response dependence of NF–kB activation contrasted with the flat dependence in the antibody production test. This discrepancy may be explained by influence on adjuvant activity of such side effect as pyrogenicity. Indeed, GMDP-Lys is the most pyrogenic compound among the GM-containing glycopeptides (unpublished data). It is likely, therefore, that the

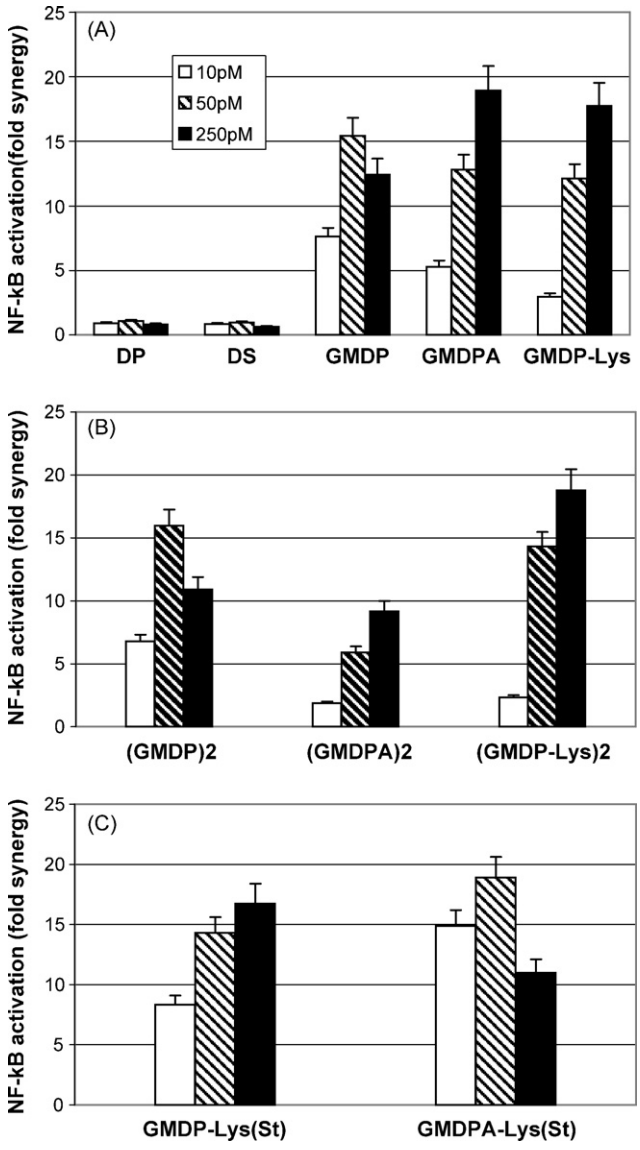


Fig. 3. NOD2 activation by glycopeptides. Human HEK293 epithelial cells were co-transfected with expression vectors for NOD2 in the presence of increasing concentration of: (A) disaccharide glycopeptides, DS-disaccharide, DP-dipeptide; (B) tetrasaccharide glycopeptides and (C) disaccharide glycopeptides with ε-stearoyl-lysine and the activity of NF–kB-driven luciferase reporter gene was measured. Data are presented as fold activation over NF–kB activation induced by each individual construct. Data show the mean ± S.E.M of duplicates.

pyrogenic effect at high peptide doses inhibited the antibody production.

The high adjuvant activity of "dimeric" $(GMDP\text{-}Lys)_2$ and $(GMDP)_2$ correlated well with the high values of NF–kB activation and the similarity of dose–response dependencies. Interestingly, within the second group, $(GMDPA)_2$ showed the lowest adjuvant activity and NOD2 stimulation. In addition, comparison of $(GMDPA)_2$ and GMDPA activities in vitro and in vivo pointed to less efficient "dimer" – NOD2 than "monomer" – NOD2 interaction in case of these deamidated derivatives.

Stearoyl containing muramyl peptides have been synthesized with the aim of improving their interaction with cell membrane and intra cell delivery. Indeed, the relatively smooth dose–response dependencies of NF–kB activation by peptides from that group can be explained by enhanced affinity of stearoyl containing molecules to the NOD2. However, stimulation of antibody production by these peptides shows no clear evidence of the expected enhanced interaction of Lys(St) containing analogs with the cell membrane or intracellular delivery. Thus, GMDP-Lys(St) demonstrated intermediate stimulation indexes and GMDPA-Lys(St) was not significantly more active than the parent non-acylated molecules (1) and (3). Possibly, the respective in vivo effects are masked by increased toxicity of these compounds (LD_{50} = 375 mg/kg for GMDP-Lys(St) versus LD_{50} = 7000 mg/kg for GMDP, unpublished data).

In summary, our results indicate that the main adjuvant activity in vivo of the examined muramyl peptides satisfactorily correlates with the observed in vitro NF–kB activation through NOD2. This observation accords with the conclusion of Kobayashi et al. [15] that "NOD2 is able to activate adaptive immunity and mediate adjuvant activity in the production of the antibody to T-cell dependent antigen". It is also apparent that the adjuvant activity of muramyl peptides is influenced by certain side effects of these compounds, including toxicity and pyrogenicity. Additionally, another cellular pathway may exist, which is responsible for the minor adjuvant effect of muramyl peptides at low concentration ranges.

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