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Biochemical characterization of glucosaminylmuramyldipeptide binding sites of murine macrophages

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Abstract By using radioligand analysis, murine peritoneal macrophages were shown to express several hundred high-affinity cell surface GMDP-binding sites (K_a 350 pM). Photoaffinity labeling followed by SDS-PAGE enabled us to identify 32–34 and 38 kDa proteins inside these cells that bound GMDP specifically.

Key words: Muramyl peptide receptor; Affinity labeling; Radioligand analysis

1. Introduction

N-Acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-D-isoglutamine (GMDP) is a fragment of bacterial cell wall demonstrating a variety of biological activities [1]. It belongs to a large group of muramylpeptides (MPs) known to markedly affect the immune response. It's adjuvant and anti-tumor effects, and ability to induce resistance to bacterial and viral infections are well documented [1,2]. Despite of large number of studies the molecular mechanism of the MPs' biological activity remains uncertain. Most likely their activity is mediated by receptor mechanisms. This hypothesis is supported by stereospecificity of action as well as by a saturating dose–response relationship [3,4].

MP-binding sites have been found on macrophages and other cells [5,6], but conflicting data were published regarding their location. Silverman et al. found a low number of muramyldipeptide (MDP) receptors (several hundred per cell) on the surface of murine macrophages [6]. According to other investigators MP-binding sites are located inside macrophages and their numbers comprise dozens or hundreds of thousand per cell [5,7,8].

In this study we used ^{125}I-labeled derivatives of GMDP in order to characterize both external and internal GMDP-binding sites of murine peritoneal macrophages.

2. Materials and methods

Female Balb/c mice were purchased from the breeding house of Russian Medical Academy of Sciences (Stolbovaya, Moscow Region). GMDP and its derivatives were synthesized in the Laboratory of Peptide Chemistry, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, as described in [9]. ^{125}I-labeled GMDP derivative, GMDP-Lys ([^{125}I]Hp) (2000 Ci/mmol), synthesized as in [10,11], was a kind gift of A. Kaidalov (Engelgart Institute of Molecular Biology, RAS, Moscow). Streptavidin–horseradish peroxidase conjugate was a kind gift of Dr. V. Kovalenko (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, RAS).

2.1. Synthesis of GMDP-Lys azidosalicilic acid, GMDP-Lys(AzS)

The *N*-hydroxysuccinimide ester of 4-azidosalicilic acid (NHS-AzS) was synthesized as described in [12]. A dioxane solution of NHS-AzS (250 μg in 25 μl) was added to 800 μg of GMDP-Lys in 200 μl 0.05 M phosphate buffer, pH 7.5. The reaction mixture was stirred for 30 min at room temperature in the dark. The conjugate was purified by reverse-phase HPLC using Nucleosil 7C$_8$ column (4 × 120 mm, Macherey-Nagel). The column was equilibrated with 10% aqueous acetonitrile, containing 0.05% trifluoroacetic acid (TFA). Fractions containing GMDP-Lys(AzS) were liophilized. The purity of the conjugate was proved by thin-layer chromatography on silica gel plates (Eastman-Kodak) using methanol/acetone, 1:10, as the mobile phase. Spots were visualized under a UV lamp.

2.2. Radioiodination of GMDP-Lys(AzS)

GMDP-Lys(AzS) (50 μg) in 50 μl of 0.05 M phosphate buffer, pH 7.5, was placed in a vial along with 75 μg NaI, containing 0.09% of ^{125}I (1 mCi). Chloramine T (1% solution in deionized water, 10 μl) was added. The reaction was stopped 30 s later by the addition of 10 μl 1% aqueous solution of sodium thiosulphate. Iodinated conjugate was purified by HPLC on a Nucleosil 7C$_8$ column (Macherey-Nagel), equilibrated with 10% aqueous acetonitrile, containing 0.05% TFA. Elution was carried out with a 10–60% acetonitrile gradient in aqueous 0.05% TFA. Fractions (1 ml) were collected, and radioactivity measured using a Compugamma counter (LKB). Fractions containing GMDP-Lys([^{125}I]AzS) were lyophilized. The specific radioactivity of GMDP-Lys([^{125}I]AzS) was 3 × 10^3 cpm/min/pmol.

2.3. Isolation of mirine peritoneal macrophages

Mice were killed by cervical dislocation. Peritoneal exudate cells were washed with Dulbecco's PBS, supplemented with 5% fetal calf serum (FCS), and incubated for 1 h in plastic Petri dishes at 37°C in 5% CO_2. Non-adherent cells were washed out with Dulbecco's PBS. Macrophages were removed from the plastic surface with a cell scrapper.

2.4. Analysis of kinetics of GMDP-Lys([^{125}I]Hp) binding to viable macrophages

GMDP-Lys([^{125}I]Hp) (2 × 10^6 cpm, 0.45 nmol) in 10 μl PBS, was added to 5 × 10^5 viable macrophages in 100 μl PBS, containing 1% BSA. The incubation was carried out for 0–2 h at 4°C. Aliquots were removed from the incubation mixture every 2.5 min and filtered through Whatman GF/F filters which had been soaked for 12 h at 4°C in 25 mM MOPS-KOH, 1 mM $MgSO_4$ buffer, pH 7.4 (buffer A), containing 1% BSA, for reduction of non-specific binding. Each filter was washed three times with 3 ml of the same buffer and the radioactivity counted using Compugamma counter.

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Abbreviations: MP, muramyl peptide; MDP, MurNAc-Ala-D-iGln; GMDP, GlcNAc-β1-4-MurNAc-Ala-D-iGln; GMDP-Lys, GlcNAc-β1-4-MurNAc-Ala-D-iGln-Lys; PBS, phosphate buffered saline; BSA, bovine serum albumin; Hp, 3-(4-hydroxyphenyl)propionyl; AzS, 4-azidosalicyl.

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For evaluation of non-specific binding, 140 nmol of unlabeled GMDP were added to macrophages along with GMDP-Lys([^{125}I]Hp). Specific binding was calculated by subtracting non-specific binding from the total binding. The standard deviation was calculated as in [13].

2.5. Dose-dependence of GMDP-Lys([^{125}I]Hp) binding to viable macrophages

Increasing amounts of labeled ligand (67.5 pmol–2.25 nmol) were added to 5×10^5 viable cells in 100 μl PBS, containing 1% BSA, and 0.01% sodium azide. Unlabeled GMDP (1000-fold excess) was included in control samples. Incubation was carried out for 30 min at 4°C. Cells were harvested onto Whatman GF/F filters, washed three times with buffer A containing 0.1% BSA, and the radioactivity counted using Compugamma counter.

2.6. Inhibition analysis of GMDP-Lys([^{125}I]Hp) binding to viable macrophages

10 μl (2×10^6 cpm, 0.45 nmol) GMDP-Lys([^{125}I]Hp) in PBS was added to 5×10^5 macrophages in 100 μl PBS, containing 1% BSA, 0.01% sodium azide. Increasing amounts (0.01–10 μmol) of inhibitor (GMDP, GMDP-Lys or L-GMDP) were added simultaneously with the radiolabeled ligand. The incubation was carried out for 30 min at 4°C. Cells were harvested onto GF/F filters and washed three times with buffer A containing 0.1% BSA. Radioactivity was counted using a Compugamma counter.

2.7. Photoaffinity labeling of GMDP-binding molecules

Macrophages (10^6 cells) were permeabilized with 0.001% digitonin in Dulbecco's PBS for 1 min at 20°C and washed 3 times with the same buffer. Cells were suspended in 1 ml Dulbecco PBS and incubated in plastic Petri dishes with 3.3 nmol (10^7 cpm) of GMDP-Lys([^{125}I]AzS) for 30 min at 37°C in the dark. Petri dishes were placed in the refrigirator for 5 min and irradiated for 10 min at a distance of 15 cm by a Black Ray CL-215 (UV Products Inc.). Control samples contained, along with GMDP-Lys([^{125}I]AzS), unlabeled GMDP (1000–10,000 excess) or GMDP-Lys(AzS) for evaluation of non-specific binding.

2.8. Gel-electrophoresis

SDS-PAGE was carried out in 12% slab gels according to Laemmli [14]. Cells were harvested and pelleted by centrifugation at $400 \times g$ for 10 min at 4°C. The cell pellet was lysed with hot sample buffer and boiled for 5 min. After electrophoresis, gels were stained with Coomassie R-250, destained, dried and processed for autoradiography for 5–15 days at −70°C using RT-6M film. The autoradiographic films were scanned using a 2202 Ultroscan Laser Densitometer (LKB).

3. Results and discussion

In our earlier studies we failed to detect GMDP-binding sites on macrophage plasma membranes. Taking in account results obtained for muramyldipeptide (MDP) by Silverman et al. [6] this could be due to insufficient sensitivity of our method, namely flow cytometry. Using this method we could detect receptor molecules only if their number exceeded 1000 per cell. This value is higher than the number of MDP receptors found by Silverman.

In order to increase the sensitivity of the assay in this study we used radioligand analysis. It was performed with the aid of a ^{125}I-labeled derivative of GMDP-lysine, namely GMDP-Lys([^{125}I]Hp) (Fig. 1). Not long ago we showed that GMDP-Lys and its derivatives, substituted at the ε-amino group of lysine, had biological activities identical to that of GMDP [15]. It enabled us to use these derivatives, in particular GMDP-Lys([^{125}I]Hp) and GMDP-Lys([^{125}I]AzS), in receptor-labeling studies.

Plasma membrane GMDP-binding sites were detected by incubating viable macrophages with GMDP-Lys([^{125}I]Hp) on ice, in the presence of sodium azide in order to prevent endocytosis. To differentiate specific and non-specific binding, inhibition analysis was carried out. Unlabeled GMDP (1000-fold excess) was used as an inhibitor. The difference between binding in the absence and the presence of unlabeled GMDP was considered as specific binding.

A kinetic study of GMDP-Lys([^{125}I]Hp) binding to murine peritoneal macrophages has shown that total binding increased upon an increase in incubation time, whereas specific binding remained almost unchanged already after 5 min incubation (data not shown). It should be noted that the level of non-specific binding was high. A similar high level of non-specific binding of radiolabeled MDP to cells was observed by Silverman et al. [6].

Specificity of binding was confirmed by dose-dependent inhibition by GMDP and GMDP-Lys (Fig. 2). A GMDP analog

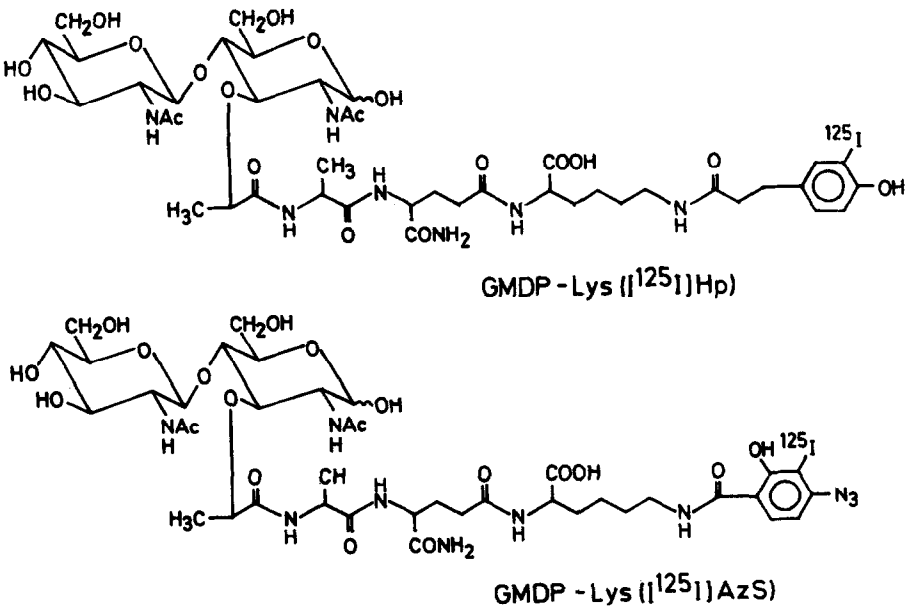

Fig. 1. Radiolabeled derivatives of GMDP.

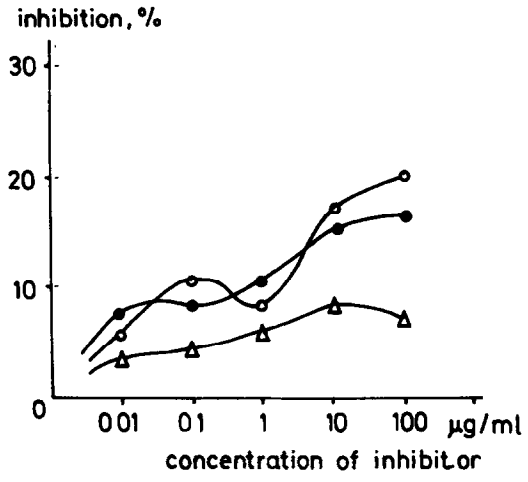

Fig. 2. Inhibition analysis of GMDP-Lys([^{125}I]Hp) binding to viable macrophages. Inhibition with GMDP (●), GMDP-Lys (○), L-GMDP (△).

with the L-configuration of the isoglutamine residue (L-GMDP) was a much less effective inhibitor, demonstrating a correlation with the absence of appreciable biological activity.

Scatchard analysis of binding revealed (Fig. 3) that there were nearly 1000 plasma membrane GMDP-binding sites per cell with a dissociation constant of 350 pM. It should be noted that the K_d value coincided with that determined by Silverman et al. for MDP [6], where as the B_{max} was almost two-times higher [17].

The role of plasma membrane MP-binding sites remains uncertain. The data obtained by several groups indicated that to produce a biological effect, MPs have to be internalized by macrophages [7,16]. An intracellular MDP-binding protein with a molecular weight of 40–45 kDa was identified by Tenu et al. in rabbit alveolar macrophages [8]. Two types of GMDP-binding sites, with K_d's of 20 and 540 nM, were reported by Sumaroka et al. to exist inside murine peritoneal macrophages [5].

To further characterize these binding sites, namely to identify

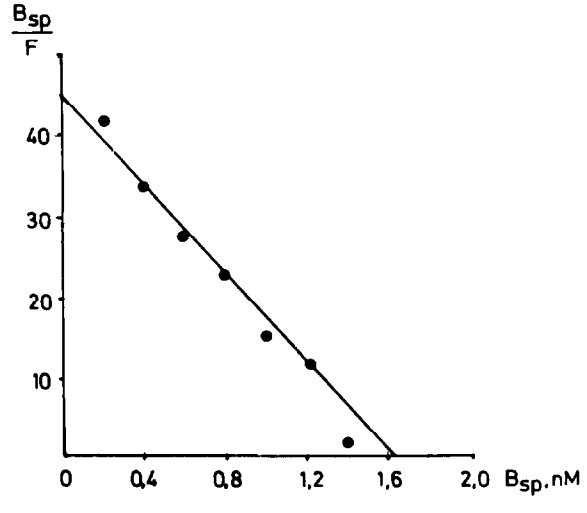

Fig. 3. Scatchard plot of binding of GMDP-Lys([^{125}I]Hp) to viable macrophages.

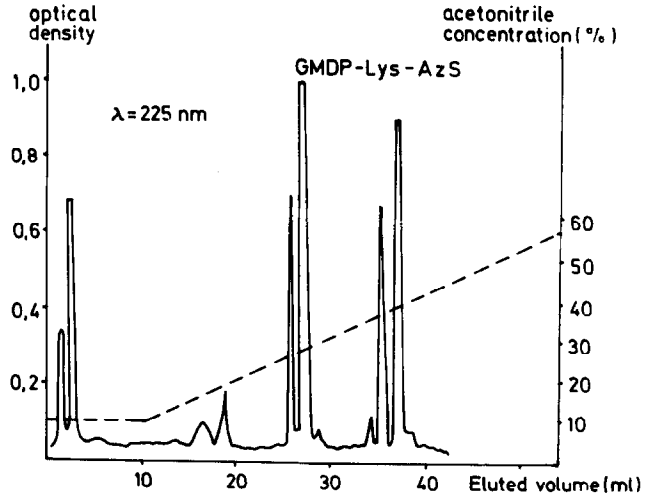

Fig. 4. Reverse-phase HPLC of GMDP-Lys(AzS).

GMDP-binding proteins, we used the affinity labeling technique. To covalently link GMDP to receptor proteins, a photoactivated derivative was synthesized. The 4-azidosalicilic acid residue (AzS) was coupled to GMDP-Lys by use of succinimide ester [10]. The conjugate GMDP-Lys(AzS) (Fig. 1) was purified by reverse-phase HPLC (Fig. 4): the dashed peaks on the chromatogram, eluted at 26–28% acetonitrile, represent two GMDP anomers known to be always present in aqueous solutions [11]. These two fractions were combined and subjected to radioiodination by the chloramine T procedure [10]. Purification of radiolabeled GMDP-Lys([^{125}I]AzS) was achieved by reverse-phase HPLC.

Digitonin-permeabilized macrophages were incubated with radiolabeled ligand at 37°C in order to facilitate GMDP-Lys([^{125}I]AzS) uptake by cells. Specificity of binding was evaluated by introducing 1000–10,000 fold excess of unlabeled GMDP along with GMDP-Lys([^{125}I]AzS). Only around 40% of label was shown to bind specifically, indicating a high level of non-specific binding, obviously due to the iodoazidosalicilic acid residue. Nonetheless, GMDP competed with GMDP-Lys([^{125}I]AzS) for internal binding sites, enabling us to use the above-mentioned derivative for photoaffinity labeling of macrophages.

After treating peritoneal macrophages with digitonin, permeabilized cells were incubated with GMDP-Lys([^{125}I]AzS) in the absence or presence of unlabeled GMDP, washed several times with PBS and irradiated with UV. Cells were lysed with hot sample buffer and subjected to SDS-PAGE. After drying the gel, radiolabeled proteins were visualized by autoradiography.

Affinity labeling demonstrated the presence of several GMDP-binding proteins (Fig. 5). Binding of radioligand to 32–34 and 38 kDa proteins could be completely inhibited by unlabeled GMDP, indicating specificity, whereas binding to the 43 kDa protein could be inhibited only partially. Complete inhibition of binding to the 43 kDa protein was achieved by using a 100 fold excess of non-iodinated GMDP-Lys(AzS) conjugate. Thus, most likely, various parts of the GMDP-Lys(AzS) molecule contribute to its binding to the 43 kDa protein. In contrast to our finding, Tenu et al. reported that binding of a [^{125}I]AzS derivative of MDP to an internal 40–45 kDa receptor of rabbit alveolar macrophages could be completely inhibited

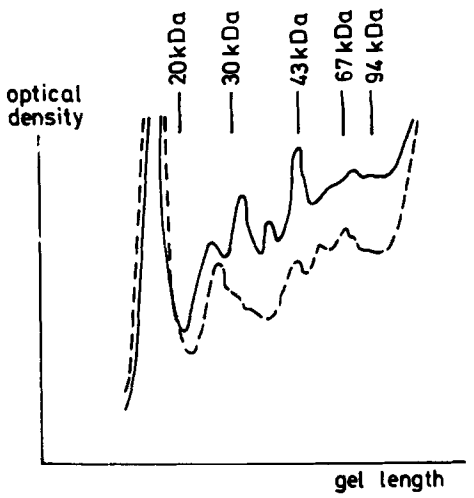

Fig. 5. Scan from the autoradiography film from SDS-PAGE of GMDP-Lys([^{125}I]AzS)-labeled permeabilized peritoneal macrophages.

by MDP [8]. At the moment we can not answer the question of whether the 43 kDa MP-binding protein in murine macrophages and the 40–45 kDa protein in rabbit macrophages perform identical functions.

An attempt to use the same approach to affinity label plasma membrane GMDP-binding proteins failed, evidently due to the low number or receptors and low yield of the coupling reaction (less than 5%).

Thus, the most probable intracellular targets of GMDP in murine macrophages are the 32–34 and 38 kDa proteins. The same proteins were labeled when myelomonocytic WEHI-3 cells were studied (data not shown). Sequencing of these proteins is under way.

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