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Muramyl peptide-binding sites are located inside target cells

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Using flow cytometry and fluorescence polarization analysis, specific muramyl peptide-binding sites were shown to be located inside T-lymphocytes, macrophages and neuroblastoma cells, but not inside B-cells. No binding sites were found on the cell surface. The number of binding sites for each cell type was determined. Two types of binding sites were observed for myelomonocytic WEHI-3 cells with K_d values of 21 and 540 nM. Inhibition analysis demonstrated that for effective binding, an intact glycopeptide molecule and D-configuration of isoglutamine residue are important.

Muramyl peptide-binding site; Mammalian cell; Flow cytometry

1. INTRODUCTION

Structural components of the bacterial cell wall – muramic acid-containing glycopeptides (MPs) – produce multiple biological effects on the immune and central nervous systems of mammals [1,2]. They demonstrate adjuvant activity, cause growth inhibition and necrosis of certain tumours, and induce non-specific resistance to bacterial and viral infections.

Despite of a large number of studies dealing with muramyl peptides, the molecular mechanism of their activity remains unclear. Different conclusions were reached concerning the presence of MP-binding molecules on target cells [3–5]. Only recently the presence of these molecules was demonstrated for macrophages and B-cells, though their location (on the plasma membrane or inside the cells) is still under discussion [6,7].

In the present study, by means of flow cytometry and fluorescence polarization analysis, the presence and location of specific MP-binding sites for cells of various lineages were investigated.

2. MATERIALS AND METHODS

2.1. *Cells and cell lines*

Macrophages were isolated from peritoneal cells of Balb/c mice by adherence to plastic Petri dishes as in [8]. Normal B- and T-cells were isolated from spleens of C57BL/6 mice. Macrophages were depleted by adherence to plastic. Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 0.05 mM 2-mercaptoethanol.

Abbreviations: MP, muramyl peptide; GMDP, GlcNAc-β1-4-MurNAc-alanyl-D-isoglutamine; L-GMDP, GlcNAc-β1-4-MurNAc-alanyl-isoglutamine; GMDP-Lys, GlcNAc-β1-4-MurNAc-alanyl-D-isoglutamyl-lysine; PBS, phosphate-buffered saline.

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2.2. *Synthesis and FITC-labelling of muramyl peptides*

Disaccharide-containing muramyl peptides (DMP) used in this study were synthesized as described earlier [9]. FITC was conjugated to GMDP-L-lysine (GMDP-Lys) as in [10]. Resulting fluoresceinthiocarbamoyl-Lys-GMDP (GMDP-Lys-FluTC) was isolated by reverse-phase HPLC on a Zorbax ODS column (0.4 × 24 cm, DuPont, USA) using an acetonitrile gradient (0–60%) in 0.05 M ammonium acetate buffer, pH 6.0.

2.3. *Permeabilization of cells*

Cells (5×10^5 per ml) were fixed for 10 min with 4% paraformaldehyde in PBS, pH 7.4, and permeabilized by treating with 0.2% β-octyl glucoside in PBS for 5–10 min.

2.4. *Cell staining and flow cytometry*

GMDP-Lys-FluTC (10 μg) and various doses of inhibitors ranging from 0.01 to 100 μg were added simultaneously to 5×10^5 permeabilized cells in 100 μl PBS containing 1% bovine serum albumin (PBS-BSA). Incubation was carried out for 30 min at 4°C in the dark. Viable cells were treated similarly in an ice bath with GMDP-Lys-FluTC in PBS-BSA containing 0.01% sodium azide. After incubation, cells were washed twice with PBS-BSA and once with PBS.

Double immunofluorescent staining of spleen cells (5×10^5) with GMDP-Lys-FluTC and phycoerythrin-labelled monoclonal anti-L3T4 antibody (Beckton-Dickinson, USA) or rhodamin-labelled anti-mouse Ig antibodies (DAKO Parts, Denmark) was carried out for 30 min at 4°C in PBS-BSA-sodium azide.

Flow cytometer EPICS 'Elite' (Coulter, USA) was used. The data were evaluated using the Multigraph program (Coulter, USA).

2.5. *Fluorescence polarization analysis*

GMDP-Lys-FluTC (10 μg) and various doses of inhibitors ranging from 0.01 to 100 μg were added to 7.5×10^5 permeabilized cells in 1.5 ml PBS.

Fluorescence polarization was measured on Hitachi-MPF4 spectrofluorimeter (Hitachi, Japan) in quartz cells (10 × 10 mm) at 37°C by the standard procedure [11] taking into account the light scattering of the cell suspension (excitation wavelength, 490 nm; emission wavelength, 515 nm).

The fraction of bound ligand and K_d values were determined accord-

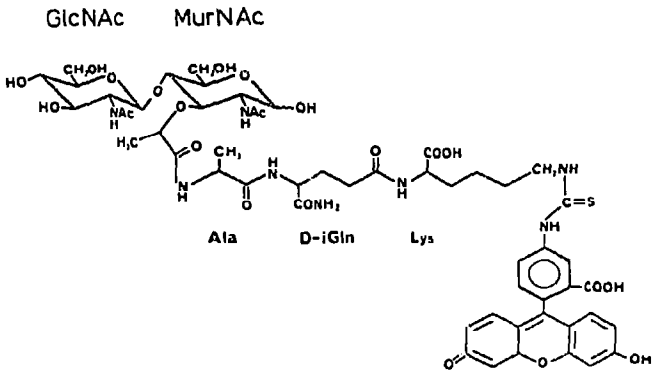

Fig. 1. Fluorescent ligand (GMDP-Lys-FluTC) used in the study.

ing to Lakowitcz [12]. Dissociation constants were calculated from a Scatchard plot [13].

3. RESULTS AND DISCUSSION

Among possible molecular mechanisms of biological activity of MPs, the receptor mechanism seems to be the most probable [14,15], although conflicting data has been published on this subject [3–7]. In order to clarify this question, a fluorescein-labelled derivative of GMDP-Lys was synthesized with a fluoresceinthiocarbomoyl residue attached to the ε-amino group of lysine (Fig. 1). This derivative had immunostimulatory activity comparable to GMDP and GMDP-Lys, causing Ia antigen induction on murine peritoneal macrophages.

Binding of GMDP-Lys-FluTC to normal cells and cell lines was studied by using 2 methods, namely flow cytometry and fluorescence polarization analysis. Both approaches gave identical results. No specific binding sites were found on the cell plasma membrane. In contrast, a large number of MP-binding sites was found inside all cells studied, although specific binding could be demonstrated for only murine macrophages and myelomonocytic cells (WEHI-3), human neuroblastoma cells (IMR-32), human (Jurkat) and rat (LSK) T-cells, but not for murine myeloma (SP2/0, PAI) and lymphoma (EL-4) cells (Table I).

The presence of specific DMP-binding sites for T-, but not for B-cells was confirmed by using two-colour flow cytometric analysis of permeabilized macrophage-depleted murine spleen cells (see Fig. 2), stained with either phycoerythrin-labelled anti-L3T4 (T-helper marker) antibody or rhodamin-labelled anti-Ig antibodies (B-cell marker) plus GMDP-Lys-FluTC. For both phycoerythrin- and rhodamin-labelled cells, binding of GMDP-Lys-FluTC was observed, but only for phycoerythrin-labelled cells could this binding be inhibited by GMDP (Fig. 2C).

Our results obtained for murine peritoneal macrophages agree with the data obtained by Tenu et al. [7]

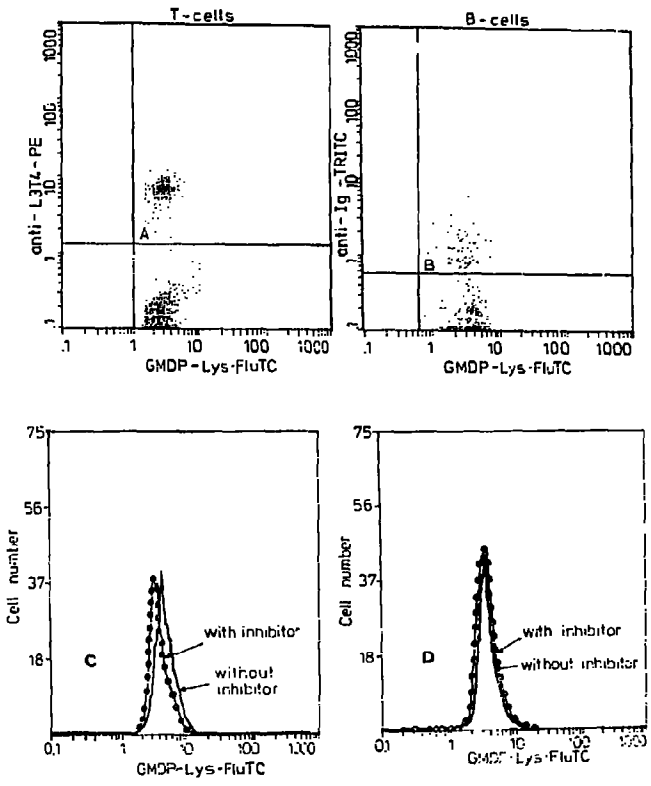

Fig. 2. Flow cytometric analysis of GMDP-Lys-FluTC binding to murine spleen T- and B-cells. A,B: in the absence, C,D: in the presence of uniabelled GMDP.

for rat alveolar macrophages. In contrast Silverman et al. [6] have found MP-binding sites on the plasma membrane of murine macrophages. Our data do not exclude the presence of a low number of MP-binding sites on the cell surface, because the fluorescent label used in this study enables the detection of not less than 1000 binding sites per cell. Consequently, the several hundred specific

Table I

Number of MP-binding sites for various cells and cell lines estimated by flow cytometry

Cells	Number of binding sites*			
	Permeabilized cells		Viable cells	
	Total	Specific	Total	Specific
Macrophages	150000	98000 (65%)	<2000	<1000
WEHI-3	88000	30350 (35%)	4300	<1000
JURKAT	179900	40700 (23%)	9700	<1000
EL-4	135300	none	<2000	<1000
LSK	132500	32800 (25%)	2700	<1000
PAI	69700	none	<2000	<1000
Sp2/0	35900	none	<2000	<1000
IMR-32	593200	75000 (13%)	<2000	<1000

*Average number out of at least 3 experiments

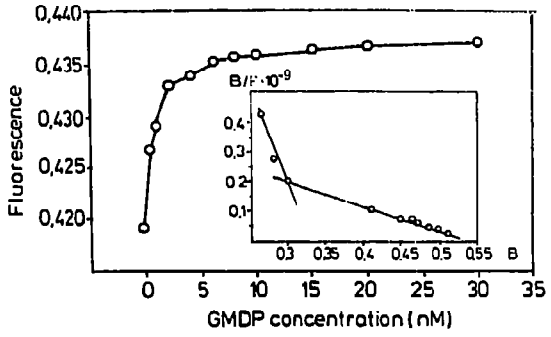

Fig. 3. Fluorescence polarization analysis of binding of GMDP-Lys-FluTC to WEHI-3 cells. *Inset*, Scatchard plot of the data.

MP-binding sites per cell as observed by Silverman et al. [6] could remain undetected.

Fluorescence polarization data for WEHI-3 cells revealed the presence of 2 types of DMP-binding sites with K_d values of 21 and 540 nM (Fig. 3). The affinity of ligand binding is much lower than described [6] for resident macrophages (48 pM). Again we cannot exclude the presence of a low number of similar receptors on WEHI-3 cells, because the sensitivity of the method used was not high enough for their detection.

Inhibition analysis showed that an intact GMDP structure as well as the stereochemistry of the molecule are essential for effective binding; the disaccharide GlcNAc-MurNAc, tripeptide Ala-D-iGln-Lys and the GMDP stereoisomer with a D-Igln residue substituted for the L-iGln (L-GMDP) were much less efficient inhibitors than GMDP or GMDP-Lys (Fig. 4). Taking into account that these compounds are biologically inactive, the above data show a correlation between ligand specificity of binding and ligand specificity for biological activity.

Thus, the MP-binding sites are located inside macrophages, T-helper cells and nerve cells. Evidently the ability of these cells to respond to MP depends on their ability of uptake of the glycopeptide. This process is easiest for phagocytic cells, e.g. macrophages. Our results correlate with functional studies showing that for activation of macrophages, MPs have to get inside the cell [16,17].

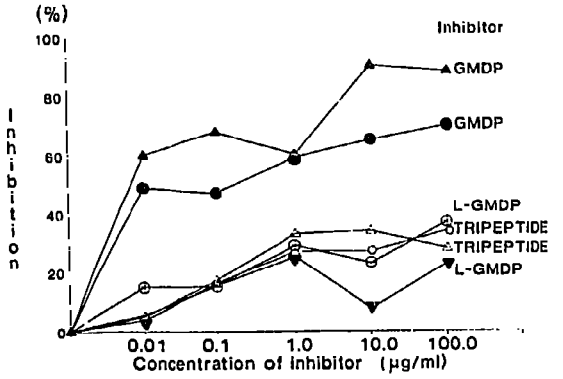

Fig. 4. Inhibition of GMDP-Lys-FluTC binding to murine peritoneal macrophages (circles) and WEHI-3 cells (triangles) by GMDP, L-GMDP and tripeptide Ala-D-iGln-Lys. Inhibition is represented as percent of GMDP-Lys-FluTC-stained cells which changed their fluorescence in the presence of inhibitor.

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