

Muramyl peptides bind specifically to rat brain membranes

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The capability of immunoactive muramyl peptides to bind specifically to rat brain membranes has been discovered. The reaction of an *N*-acetylglucosaminylmuramyl dipeptide analog having an additional C-terminal lysine residue (GMDP-Lys) with Bolton-Hunter reagent or *N*-hydroxysuccinimidyl-4-azidosalicylate afforded two acylated derivatives, GMDP-Lys(Hp) and GMDP-Lys(Azs). Their iodination resulted in radioactive derivatives (spec. act. ~ 2000 Ci/mmol) whose binding to rat brain membranes is characterized by $K_d \sim 3$ nM and $B_{\max} \sim 10$ fmol/mg membrane protein. Binding could be inhibited by muramyl dipeptide (MDP), GMDP-Lys, and GMDP, while fragments of the latter, *N*-acetylglucosamine, dipeptide and disaccharide, were ineffective.

Muramyl peptide; Specific binding; (Rat brain)

1. INTRODUCTION

Muramyl peptides, the bacterial cell wall components, manifest a variety of biological activities that underlie the practical significance of both naturally occurring compounds and synthetic preparations [1]. The immunostimulatory and pyrogenic properties of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP), a structural component of mycobacteria, are studied best in Freund's complete adjuvant [2]. MDP and some analogs, including those with an *N*-acetylglucosamine moiety, prolong slow wave sleep and can be considered to be endogenous somnogenic factors [3]. Such activity implies the presence of a specific target in the brain, however, there are

yet no biochemical data available on the respective binding sites.

Here, we report on the preparation of radioactive analogs of *N*-acetylglucosaminyl($\beta 1 \rightarrow 4$)-*N*-acetyl-L-alanyl-D-isoglutamine, a compound previously studied in detail at our institute [4,5], and describe their detection with the aid of the specific binding sites for muramyl peptides in rat brain membranes.

2. MATERIALS AND METHODS

MDP was prepared as in [6], Bolton-Hunter reagent [7] was obtained from Fluka (Switzerland), Na^{125}I from Isotop (USSR), and chloramine T, BSA and Taps from Serva (FRG). The photoactivatable derivatives were irradiated on a Chromatoscop instrument at $\lambda_{\max}$ 254 nm, samples being placed 5–10 cm from the light source. Radioactivity was measured on a Compugamma (LKB) scintillation counter. Binding data were treated by the least-squares procedure using a program (in BASIC) developed for an Iskra-226 computer. Binding parameters were calculated as in [8].

2.1. GMDP-Lys(Hp)

The GMDP-Lys [9] solution (2 mg, 2.2 μmol) in 200 μl water was mixed with Bolton-Hunter reagent (1.5 mg, 5.8 μmol) in 150 μl MeOH and then added to 200 μl of 0.05 M sodium borate buffer, pH 8.5. After incubation of the reaction mixture for 30 min at room temperature, the desired product was isolated by HPLC (fig. 1a).

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Abbreviations: Azs, 4-azidosalicyl; BSA, bovine serum albumin; GMDP, *N*-acetylglucosaminylmuramyl dipeptide; GMDP-Lys, GMDP-Lys(Azs) and GMDP-Lys(Hp), GMDP analogs with free or acylated lysine side chain; Hp, 3-(4-hydroxyphenyl)propionyl; MDP, muramyl dipeptide; Taps, *N*-tris(hydroxymethyl)-3-aminopropanesulfonic acid

2.2. GMDP-Lys(Azs)

250 μ g (906 nmol) *N*-hydroxysuccinimidyl-4-azidosalicylate [10] in 25 μ l dioxane was added to GMDP-Lys (800 μ g, 900 nmol) in 200 μ l sodium phosphate buffer (pH 7.5). The mixture was incubated at room temperature for 30 min and then separated by HPLC (fig.1b).

2.3. GMDP-Lys(125 I]Hp)

A solution of GMDP-Lys(Hp) (45 μ g, 40 nmol) in 15 μ l of 0.05 M sodium phosphate buffer (pH 7.5), 0.5 mCi Na 125 I (2000 Ci/mmol) in 4 μ l water, and 150 μ g (535 nmol) chloramine T in 15 μ l of 0.05 M sodium phosphate buffer (pH 7.5) were added to 15 μ l of 0.25 M sodium phosphate buffer (pH 7.5). In 1 min Na $_2$ S $_2$ O $_5$ (200 μ g, 1 μ mol) in 20 μ l of the same buffer was added to the mixture that was subjected to HPLC 1 min later (fig.2a).

GMDP-Lys(125 I]Azs) was prepared and purified similarly to GMDP-Lys(125 I]Hp).

2.4. Membrane isolation

Lysed membranes from rat brain were prepared essentially as in [11]; the pellet obtained after the final centrifugation was resuspended in 25 mM Taps-KOH (pH 7.4) containing 1 mM MgSO $_4$, then frozen and stored at -70° C. Protein concentration in the membrane suspension was measured by the method of Petersen [12].

2.5. Binding assays

Radioactive ligands were added in increasing amounts to 250 μ l membrane suspension (4 mg protein/ml) in buffer A [25 mM Taps-KOH (pH 7.4), 1 mM MgSO $_4$, 0.1% BSA]. In addition, a 10000-fold excess of GMDP-Lys was introduced into the control sample. After 2 h incubation at room temperature, the mixture was passed through GF/F filters (Whatman) preincubated for 12 h at 4° C in buffer A. Each filter was washed three times with the same buffer before assaying radioactivity.

3. RESULTS AND DISCUSSION

Treatment of GMDP-Lys with Bolton-Hunter reagent and subsequent separation of the reaction mixture (fig.1a) result in a series of peaks eluting at higher MeOH concentrations vs the starting compound (eluting at 10–12% MeOH). Fractions A and A' contain the Hp group (absorption maximum 276 nm), each giving rise, on rechromatography, to a two-peak profile identical to the original A/A' pattern. The latter is due to rapid interconversion of the anomers characteristic of GMDP and its derivatives [9]. From these data we conclude that fractions A and A' correspond to the modified glycopeptide, GMDP-Lys(Hp).

After reacting GMDP-Lys with the active ester of 4-azidosalicylic acid, the similarly paired fractions B and B' (fig.1b) were obtained. Their absorption maxima at 263 and 300 nm are

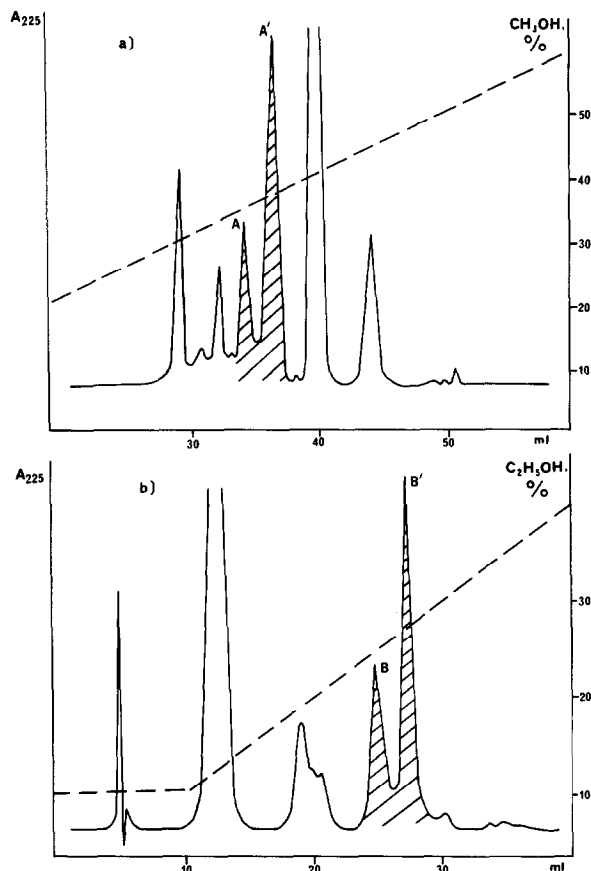


Fig.1. Separation of GMDP-Lys acylation products. (a) Modification with Bolton-Hunter reagent: Ultrasphere ODS column (0.46 $\times$ 25 cm), 0.1% ammonium trifluoroacetate buffer (pH 3.5), linear gradient of MeOH (10–50%); (b) reaction with *N*-hydroxysuccinimidyl-4-azidosalicylate: Nucleosil 7C8 column (0.46 $\times$ 25 cm), 0.1% ammonium trifluoroacetate (pH 3.5), linear gradient of EtOH (10–50%). Flow rate, 1 ml/min. Peaks A, A', and B, B', corresponding to GMDP-Lys(Hp) and GMDP-Lys(Azs), respectively, are indicated by the hatched areas.

characteristic for azidosalicylic acid, and the intensity of these bands diminishes considerably upon UV irradiation. Therefore, fractions B/B' represent the photoactivable derivative, GMDP-Lys(Azs).

In ascertaining the optimal conditions for iodination and separation of the reaction products, we first synthesized GMDP-Lys(Hp) derivatives using unlabeled NaI. Two pairs of peaks M/M' and D/D' eluted at higher MeOH concentration than peaks A/A' of the starting compound (fig.2a). With higher NaI content in the reaction

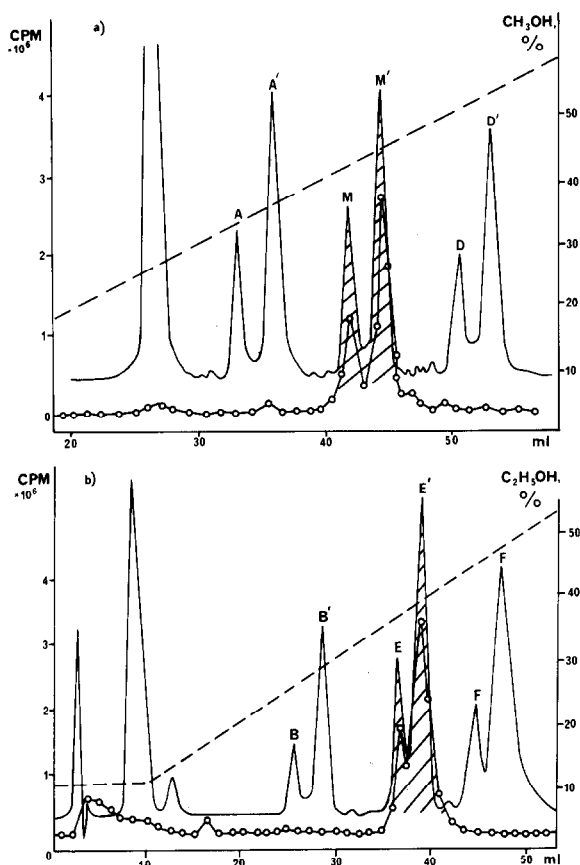


Fig.2. Isolation of GMDP-Lys iodinated derivatives. (a) Iodination of GMDP-Lys(Hp); Ultrasphere ODS column (0.46×25 cm), 0.1% ammonium trifluoroacetate buffer (pH 3.5), MeOH gradient (10–60%); (b) iodination of GMDP-Lys(Azs): Nucleosil 7C8 column (0.46×25 cm), 0.1% ammonium trifluoroacetate (pH 3.5), EtOH gradient (10–60%). Flow rate, 1 ml/min. (—) Absorption at 225 nm; (○) radioactivity.

mixture, the intensity of peaks D/D' increases at the expense of peaks A and M. The change in pH from 2.0 to 9.0 induced bathochromic shifts of $280 \rightarrow 310$ and $282 \rightarrow 315$ nm in the absorption spectra of fractions M/M' and D/D', respectively. Such behavior is also characteristic of mono- and diiodotyrosine [13]. Therefore, peaks M/M' and D/D' can be ascribed to the mono- and diiodinated derivatives of GMDP-Lys(Hp), respectively.

In order to obtain a preparation of high specific radioactivity (~ 2000 Ci/mmol), it was necessary to use undiluted Na^{125}I , thus leading to a considerable excess of GMDP-Lys(Hp) in the reaction

mixture. The radioactive product eluted similarly to peaks M/M' and, consequently, was a monoiodinated derivative.

Iodination of GMDP-Lys(Azs) resulted in two pairs of peaks (fig.2b) eluting later than peaks B/B' of the initial compound. As judged from the absorption spectra, we attribute the UV-sensitive fractions E/E' and F/F' to the mono- and diiodinated derivatives of GMDP-Lys(Azs), respectively. The radioactive fractions obtained in the reaction with undiluted Na^{125}I eluted at the position of peaks E/E', hence corresponding to the monoiodinated derivative, GMDP-Lys(^{125}I Azs).

Both GMDP-Lys(^{125}I Hp) and GMDP-Lys(^{125}I Azs) are capable of specific binding to rat brain membranes as illustrated for one of these in fig.3. The observed high level of nonspecific binding poses a serious problem. In several cases we could not reliably measure the specific binding, apparently due to some uncontrollable changes in the properties of membrane preparations.

In view of these circumstances, our main conclusion on the specific interaction of muramyl peptides with rat brain was drawn after numerous repetitions of the experiments with various batches of radioactive ligands and different membrane preparations.

The binding parameters determined from a Scatchard plot were as follows: K_d 3.1 ± 0.9 nM for GMDP-Lys(^{125}I Hp), 3.6 ± 0.3 nM for MDP-Lys(^{125}I Azs); number of binding sites $B_{\max}$ 11.1 ± 1.2 and 9.1 ± 0.9 fmol/mg protein for the Hp and Azs derivatives, respectively. The Hill coefficients h of 1.14 and 1.07 characterize one class of homogeneous binding sites.

Our results show that the binding parameters for GMDP derivatives are practically independent of the structure of the aromatic substituents at the lysine ϵ -amino group. Interestingly, the introduction of an iodoazidophenylalanine residue into MDP was found to increase the biological activity of the parent glycopeptide [14].

The interaction of GMDP derivatives with rat brain is characterized by the high affinity and extremely low density of binding sites. The latter feature may be due to the isolation of membranes from whole brain homogenates, while the respective binding centers are probably confined to a few brain structures. This idea is supported by elec-

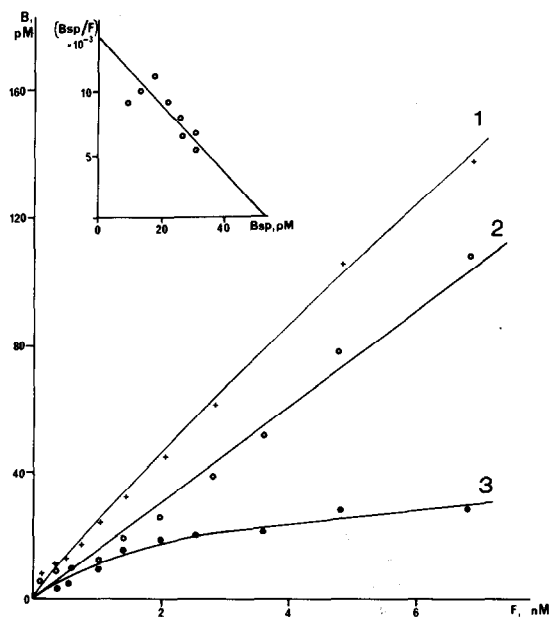


Fig.3. Total (1), nonspecific (2) and specific (3) binding as a function of GMDP-Lys($[^{125}\text{I}]\text{Hp}$) concentration (F). Membrane concentration, 4 mg protein/ml; sample volume, 250 μl ; incubation time, 2 h. (Inset) Scatchard plot.

troencephalography data on the magnitude of responses evoked by MDP administration to different substructures of rabbit brain [15].

Binding studies were carried out after 2 h incubation of radioactive ligands with membranes. Preliminary experiments showed this period to be optimal for achieving saturation. The observed slow binding correlates with the slow development of somnogenic activity upon MDP administration to brain [16].

To assess the role of structural factors in binding, we investigated the displacement of GMDP-Lys($[^{125}\text{I}]\text{Hp}$) by its fragments (table 1). GMDP, GMDP-Lys, GMDP-Lys(Hp) and MDP inhibited binding with approximately the same potency. Practically no inhibition was observed with *N*-acetylglucosamine, disaccharide and dipeptide fragments of GMDP, the value determined for the disaccharide being obviously within the limits of experimental error. Therefore, MDP is the minimal structural element required for the interaction with the brain.

In addition to muramyl peptides, other endogenous factors display somnogenic activity: δ -sleep-inducing peptide (DSIP) (review [17]),

Table 1

Displacement of GMDP-Lys($[^{125}\text{I}]\text{Hp}$) (5×10^{-9} M) with nonradioactive ligands

Ligand	Concentration (10^{-5} M)	Displacement (%)
GMDP-Lys	6.9	28 ± 5
GMDP	8.2	29 ± 5
MDP	11.6	25 ± 6
<i>N</i> -Acetylglucosamine	25.9	<1
<i>N</i> -Acetylglucosamine-($\beta 1 \rightarrow 4$)- <i>N</i> -acetylmuramic acid	11.5	6 ± 5
L-Ala-D-iGln	26.4	<1
Serotonin	14.1	<1

prostaglandin D_2 [16], and interleukin 1 [18]. Published data on receptors for these compounds in the central nervous system are sparse. Specific binding to brain was demonstrated in the case of DSIP, but the dissociation constant K_d of $14 \mu\text{M}$ [19] characterizes sites of relatively low affinity. The presence of specific binding sites for muramyl peptides on macrophages, their major targets, has been reported in [20,21]. Interestingly, the value $K_d \sim 1 \text{ nM}$ characterizing MDP-Lys interaction with macrophages [21] is close to the corresponding figure ($\sim 3 \text{ nM}$) for GMDP-Lys binding to rat brain membranes. Silverman and co-workers [21] observed competition between serotonin and MDP, and concluded that the interaction of muramyl peptides with macrophages is realized, at least in part, via serotonin receptors. The results obtained by Mašek [22] also implicate serotonergic systems in the biological activity of MDP. However, with rat brain membranes we observed no competition between serotonin and GMDP-Lys($[^{125}\text{I}]\text{Hp}$). This is indicative of certain differences between the muramyl-peptide-binding sites on brain membranes and macrophages.

Further studies with radioactive and photoactivatable analogs should help to provide a detailed characterization of the muramyl-peptide-binding sites in cells of the immune and central nervous systems.

REFERENCES

- [1] Chedid, L., Audibert, F., Lefrancier, P., Choay, J. and Lederer, E. (1976) Proc. Natl. Acad. Sci. USA 73, 2472-2475.

- [2] Lefrancier, P. and Lederer, E. (1987) *Pure Appl. Chem.* 59, 449–454.
- [3] Krueger, J.M., Davenne, D., Walter, J., Shoham, S., Kubillus, S.L., Rosenthal, R.S., Martin, S.A. and Biemann, K. (1987) *Brain Res.* 403, 258–266.
- [4] Rostovtseva, L.I., Andronova, T.M., Malkova, V.P., Sorokina, I.B. and Ivanov, V.T. (1981) *Bioorg. Khim.* 7, 1843–1858.
- [5] Ivanov, V.T., Andronova, T.M., Bezrukov, M.V., Rar, V.A., Makarov, E.A., Kozmin, S.A., Astapova, M.V., Barkova, T.I. and Nesmeyanov, V.A. (1987) *Pure Appl. Chem.* 59, 317–324.
- [6] Kusumoto, S., Tarumi, Y., Ikenaka, K. and Shiba, T. (1976) *Bull. Chem. Soc. Jap.* 49, 533–539.
- [7] Bolton, A.E. and Hunter, W.M. (1973) *Biochem. J.* 133, 529–539.
- [8] Munson, P.J. and Rodbard, D. (1980) *Anal. Biochem.* 107, 220–239.
- [9] Kaydalov, A.A., Utkin, Yu.N., Andronova, T.M., Tsetlin, V.I. and Ivanov, V.T. (1987) *Bioorg. Khim.* 13, 1523–1529.
- [10] Dupuis, G. (1987) *Can. J. Chem.* 65, 2450–2453.
- [11] Cascieri, M.A. and Liang, T. (1983) *J. Biol. Chem.* 258, 5158–5164.
- [12] Peterson, G.L. (1977) *Anal. Biochem.* 83, 346–356.
- [13] Gemmill, C.L. (1955) *Arch. Biochem. Biophys.* 54, 359–365.
- [14] Tenu, J.-P., Adam, A., Souvannavong, V., Barratt, G., Yap, A., Petit, J.-F., Level, M., Clemance, M. and Douglas, K. (1987) *FEBS Lett.* 220, 93–97.
- [15] Garsia-Arraras, J.E. and Pappenheimer, J.R. (1983) *J. Neurophysiol.* 49, 528–533.
- [16] Inoue, S., Honda, K., Komoda, Y., Uchizono, K., Ueno, R. and Hayashi, O. (1984) *Proc. Natl. Acad. Sci. USA* 81, 6240–6244.
- [17] Graf, M.V. and Kastin, A.J. (1986) *Peptides* 7, 1165–1187.
- [18] Krueger, J.M., Walter, J. and Dinarello, C.A. (1984) *Am. J. Physiol.* 246, 994–999.
- [19] Van Disk, A.M.A. and Shoenenberger, G.A. (1985) *Proc. Taniguchi Symposia on Brain Sciences No.8* (Inoue, S. and Borbely, A.A. eds) pp.167–178, Japan Sci. Soc., Tokyo.
- [20] Silverman, D.H.S. and Karnovsky, M.L. (1985) *Biochem. Biophys. Res. Commun.* 131, 1160–1167.
- [21] Silverman, D.H.S., Krueger, J.M. and Karnovsky, M.L. (1986) *J. Immunol.* 136, 2195–2201.
- [22] Mašek, K. (1988) *Adv. Biosci.* 68, 11–19.