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Specific binding of glucosaminylmuramyl peptides to histones

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Abstract Intracellular *N*-acetylglucosaminylmuramyl peptide-binding proteins of murine macrophages and myelomonocytic WEHI-3 cells were characterized. SDS-PAGE and Western blotting revealed proteins with molecular masses of 18, 32 and 34 kDa retaining the ability to specifically bind glucosaminylmuramyl dipeptide. The inhibition analysis demonstrated that only biologically active muramyl peptides but not inactive analogs or fragments of glucosaminylmuramyl dipeptide could inhibit glucosaminylmuramyl dipeptide-binding to these proteins. Purification of these proteins and sequencing of peptides obtained after in-gel trypsin digestion enabled us to identify the above mentioned proteins as histones H1 and H3. These findings suggest that nuclear histones might be target molecules for muramyl peptides.

Key words: Muramyl peptide; Receptor; Histone; Mouse macrophage

1. Introduction

N-Acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-D-isoglutamine (GMDP), the prototype of muramyl peptides [1], is a fragment of the bacterial cell wall demonstrating a variety of biological activities. Its adjuvant and anti-tumor effects, the ability to induce non-specific resistance to bacterial and viral infections are well-documented [2]. GMDP activity is mediated by a receptor mechanism. High affinity GMDP-binding sites were found on the macrophage plasma membrane as well as inside macrophages, the number of internal binding sites being two orders of magnitude higher than of membrane counterparts [3]. These internal binding sites were capable of transducing a biological signal as was demonstrated using liposome-encapsulated GMDP [4], whereas the role of membrane-binding sites remains unclear. Similar results were obtained by others upon study of muramyldipeptide (MDP), a close relative of GMDP [5–7].

Little data are available on molecular characteristics of muramyl peptide-binding proteins. Tenu et al. demonstrated the binding of a ^{125}I-labelled aryl-azide derivative of MDP to an intracellular 40–45 kDa protein in rabbit alveolar macrophages [7]. MDP was also reported by Karnovsky et al. to bind in a competitive manner to serotonin receptors on macrophages [8] and platelets [9]. The binding of soluble peptidoglycan to the human monocyte surface antigen CD14 was recently reported which could be inhibited by MDP assuming its interaction with CD14 [10]. The properties of GMDP-binding proteins of murine peritoneal macrophages were reported from this laboratory. Affinity labelling of permeabilized murine macrophages using the [^{125}I]azidosalicyl derivative of GMDP-lysine revealed that GMDP was binding specifically to 32–34 and 38 kDa proteins [11]. The former proteins were detected also on Western blots with biotinylated GMDP-containing polymeric probe, not only in murine macrophages and myelomonocytic WEHI-3 cells, but in human, rat and mouse T-helper cells as well [4]. In the present study, we report the identification of 18, 32 and 34 kDa GMDP-binding proteins (a preliminary report of this study was presented at the 13th European Immunology Meeting, June 22–25, 1997, Amsterdam [12]).

2. Materials and methods

2.1. Materials and reagents

Cell culture medium and supplements were obtained from Gibco Life Technologies (UK). Chemicals and enzymes were purchased from Sigma Chemicals (St. Louis, USA). Blotting membranes and membrane filters were obtained from Millipore (Moscow, Russia). Streptoavidin-horseraddish peroxidase conjugate was kindly provided by Dr A. Zinchenko (Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia).

2.2. Cells and cell cultures

WEHI-3 cells were grown in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum (FCS).

Murine peritoneal macrophages were purified by adsorption to plastic. Briefly, mice were killed by cervical dislocation. Peritoneal exudate cells were washed with medium 199, supplemented with 5% (v/v) FCS and incubated in the same medium for 2 h in 10 cm plastic Petri dishes. Non-adherent cells were washed out with Dulbecco's PBS. Macrophages were removed from plastic surface by a cell scraper.

2.3. Synthesis of GMDP and derivatives

GMDP and other glucosaminylmuramyl peptides were synthesized as described by Rostovtseva et al. [13].

2.4. Synthesis of GMDP-lysine (20 mol%) and biotin (5 mol%) attached to the linear polyacrylamide backbone ((GMDP-Lys)-PAA-(Bi))

The synthesis of (GMDP-Lys)-PAA-(Bi) was accomplished by conjugation of *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-D-iso-

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Abbreviations: MDP, *N*-acetylmuramyl-alanyl-D-isoglutamine; GMDP, *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-D-isoglutamine; LL-GMDP, *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-L-isoglutamine; GMDP-Lys, *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-D-isoglutaminyl-lysine; GMDP-(stearoyl)Lys, *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-D-isoglutaminyl-(N^{ε}-stearoyl)lysine; (GMDP-Lys)-PAA-(Bi), GMDP-lysine (20 mol%) and biotin (5 mol%) attached to the linear polyacrylamide backbone; (Glc)-PAA-(Bi), similar probe with D-glucose residues substituted for GMDP-Lys

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glutaminyl-lysine (GMDP-Lys) and biotin to activated acrylic acid polymer as was described by Bovin et al. [14].

2.5. Electrophoresis and Western blotting

Electrophoresis was carried out according to Laemmli in 10 or 12% polyacrylamide slab gels [15]. Proteins were blotted onto a nitrocellulose membrane using a semi-dry blotter (Ancos, Denmark) according to the instructions of the manufacturer.

Blotting onto an Immobilon membrane was carried out as in [16]. The membrane was soaked for 10 min in methanol and then in blotting buffer. The blotting was performed in 25 mM sodium bicarbonate, pH 8.5, containing 10% methanol, for 2 h at 0.8 mA/cm^2. The Immobilon membrane was stained with 0.1% Coomassie R-250 in 50% aqueous methanol for 3–5 min and destained with the same solvent.

2.6. Detection of GMDP-binding proteins on Western blots

The membranes were incubated (4°C, 18 h) in 1% BSA solution in PBS, pH 7.4, in order to block remaining protein-binding sites and then washed three times with PBS, containing 0.1% Tween-20, and three times with PBS. The same procedure was repeated between each of the remaining steps. The membranes were incubated with 1 μg/ml (GMDP-Lys)-PAA-(Bi) or the similar probe with D-glucose residues substituted for GMDP-Lys ((Glc)-PAA-(Bi)) at 22°C for 1 h and then treated with streptoavidin-peroxidase conjugate (1 μg/ml) for 1 h at the same temperature. The solution of 10 mg 4-chloro-1-naphtol, 3 mg 3,3′-diaminobenzidine and 20 μl 30% hydrogen peroxide in the mixture of 10 ml PBS and 5 ml methanol was used for visualization of the bound conjugate.

In case of inhibition analysis, equal amounts of macrophage lysate protein were applied to lanes of a slab gel. After SDS-PAGE and Western blotting, the blot was cut into strips and each strip was incubated with (GMDP-Lys)-PAA-(Bi) in the presence or absence (control) of inhibitor, namely MDP (5 mg/ml), GMDP (5 mg/ml), *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-L-isoglutamine (LL-GMDP) (5 mg/ml), *N*-acetylglucosaminyl-β1-4-*N*-acetylmuramyl-alanyl-D-isoglutaminyl-(N^{ε}-stearoyl)lysine (GMDP-(stearoyl)Lys) (5 mg/ml), disaccharide GlcNAc-MurNAc (2.5 mg/ml) or dipeptide Ala-D-iGln (2.5 mg/ml).

2.7. Solubilization of GMDP-binding proteins

Cells were lysed with hypotonic buffer (2 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride). The lysate was spun at 102 000 × *g* for 1 h. The pellet was treated with 2 M KCl (KCl extract) in the case of macrophages or 2 mM Tris-HCl, pH 7.6, with 0.01 M Na-EDTA in the case of WEHI-3 cells (EDTA extract) at 22°C. Suspensions were centrifuged at 102 000 × *g* for 1 h and supernatants were kept for further experiments.

2.8. Ion-exchange HPLC

A TSK DEAE-5PW column (LKB, Sweden) was equilibrated with 20 mM Tris-HCl, pH 7.6, containing 2 mM Na-EDTA. EDTA extract of WEHI-3 cells (2–5 mg of protein) was applied and the column was washed with the above buffer until unbound proteins were eluted. A linear NaCl gradient (0–0.5 M) was applied. Fractions were lyophilized.

To assay GMDP-binding activity, 100 ml of each fraction was analyzed. Proteins were precipitated by addition of trichloroacetic acid (TCA) to make the final concentration 10%. After 30 min incubation at 4°C, the precipitate was collected by centrifugation (10 000 × *g*, 5 min). Samples were prepared for SDS-PAGE and run in 12% slab gels. GMDP-binding proteins were detected after blotting using (GMDP-Lys)-PAA-(Bi) probe as described above.

2.9. Gel filtration

HPLC purification of macrophage KCl extract was performed on a G2000SW column (Toya Soda, Japan). The column was equilibrated with 20 mM Tris-HCl, pH 7.6, containing 2 mM Na-EDTA and 0.5 M NaCl. Fractions (1 ml) were analyzed as described for ion-exchange HPLC.

2.10. Proteinase treatment

WEHI-3 cells or peritoneal macrophages were incubated in six well plates (3–5 × 10^5 cells per well) at 37°C in an atmosphere of 5% CO_2, overnight. The adherent cells were washed with PBS and treated with 1–2 U trypsin, papain or pronase in 1 ml PBS. In the case of papain, Na-EDTA and 2-mercaptoethanol were also added (1 and 25 mM, respectively). Proteolysis was monitored by light microscopy. When cells dissociated from the plastic surface, the appropriate proteinase inhibitors were added (1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide or 10 mg/ml soybean inhibitor, respectively). Cells were sedimented by centrifugation, dissolved in Laemmli sample buffer and subjected to SDS-PAGE. GMDP-binding proteins were visualized with (GMDP-Lys)-PAA-(Bi) as described above.

2.11. Protein assay

Proteins were precipitated with TCA (5% final concentration) for 30 min. The precipitate was sedimented by centrifugation (10 000 × *g*, 5 min) and washed with 2% aqueous TCA. A sodium hydroxide solution (1 N, 0.4 ml), containing 0.05% SDS, was added and the mixture was carefully heated to solubilize the precipitate. The Lowry method was used to assess the protein content [17].

2.12. Protein sequence analysis

In order to prepare samples for N-terminal analysis, lyophilized fractions, containing GMDP-binding proteins, were dissolved in 100 μl deionized water. Methanol (100 μl) and chloroform (200 μl) were added and centrifugation was carried out at 6000 × *g* for 5 min. To collect proteins, which formed a film at the interphase, solvents were removed with the syringe. The protein film was dried, dissolved in the sample buffer and subjected to SDS-PAGE in a 12% gel. After electrophoresis, proteins were blotted onto an Immobilon membrane. The stained bands were excised from the Immobilon membrane and subjected to N-terminal sequencing using an Applied Biosystems model 470A solid-phase sequencer (USA).

For peptide mapping after SDS-PAGE, the gels were stained with Coomassie blue R-250, destained and dried. The corresponding protein bands were excised and digested in situ with trypsin according to Hellman et al. [18]. The peptides were separated by reverse phase (RP)-HPLC on a 5 mm C18 Vydac resin using a linear gradient from 98% A (0.1% TFA in water) to 92% B (90% acetonitrile in 0.1% TFA/water) in 60 min and monitored at 214 nm. The HPLC system, UV detection and column construction have been extensively described by Swiderek et al. [19]. The peptides were microsequenced on a HP G1005A sequencer (Hewlett Packard, Palo Alto, CA, USA). The sequencing system was operated using standard reagents, solvents and programs (Routine 3.0) as supplied by the manufacturer.

3. Results and discussion

When photoaffinity labelling was used to identify GMDP-binding proteins, several molecules were labelled in permeabilized macrophages, but only the binding to proteins with molecular masses of 31–34, 38 and 43 kDa was markedly inhibited by cold GMDP [11]. The 40–45 kDa protein was identified using the same technique as the target molecule for MDP in rabbit alveolar macrophages by Tenu et al. [7]. The similarity between the rabbit 40–45 kDa protein and the murine 43 kDa GMDP-binding protein was strengthened by the fact that a non-labelled azidosalicyl derivative of the corresponding muramyl peptide was a much more effective inhibitor of binding than non-modified muramyl peptide.

We found that the proteins with molecular masses of 18, 32 and 34 kDa (Fig. 1, lane 1) (further designated p18, p32 and p34, respectively) were able to specifically interact with GMDP after SDS-PAGE and Western blotting [20]. The new type of probes (GMDP-Lys)-PAA-(Bi) with multiple ligand (GMDP-Lys) and biotin residues attached to the linear polyacrylamide backbone was used to demonstrate this (for review see [21]). They enable to detect ligand-protein interaction with an extremely high sensitivity. (GMDP-Lys)-PAA-(Bi) contained 20 mol% GMDP-lysine and 5 mol% biotin residues. No bands were observed upon staining with the similar polyacrylamide-based probe (Glc)-PAA-(Bi) in which

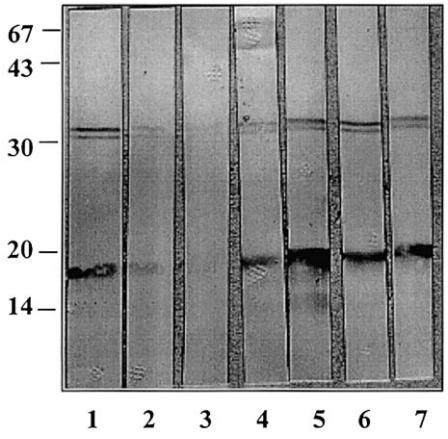

Fig. 1. Inhibition of (GMDP-Lys)-PAA-(Bi)-binding to macrophage lysate proteins on Western blots by GMDP, other muramyl peptides and GMDP fragments. Macrophage lysate was subjected to SDS-PAGE and Western blotting, followed by incubation of blots with (GMDP-Lys)-PAA-(Bi) in the presence of inhibitors, namely GMDP (lane 2), GMDP-(stearoyl)lysine (lane 3), MDP (lane 4), LL-GMDP (lane 5), GlcNAc-MurNAc (lane 6), Ala-D-iGln (lane 7). Bound (GMDP-Lys)-PAA-(Bi) was visualized with streptoavidin-peroxidase conjugate and peroxidase substrate. Lane 1, no inhibitor (control). An equal amount of lysate protein was applied to each lane.

D-glucose residues were incorporated instead of GMDP-Lys (data not shown). Therefore, GMDP-Lys rather than the PAA-(Bi) matrix was responsible for binding.

Staining the blots with (GMDP-Lys)-PAA-(Bi) in the presence of a number of GMDP analogues, namely GMDP, LL-GMDP, MDP, GMDP-(stearoyl)Lys, disaccharide GlcNAc-β1–4-MurNAc or dipeptide Ala-D-iGln, showed that only the biologically active muramyl peptides GMDP, MDP and GMDP-(stearoyl)Lys effectively inhibited the binding (Fig. 1, lanes 2–4). To obtain effective inhibition, the inhibitor had to be present in a large excess to conjugated GMDP, evidently, because of multipoint binding of the conjugate to blotted proteins which increased greatly the strength of binding. This effect is well known, e.g. for IgM antibodies. The lipophylic GMDP-(stearoyl)Lys was the most effective inhibitor. This could be due to additional non-specific interactions of the stearoyl residue with side chains of hydrophobic amino acids of the receptor proteins. GMDP was a more effective inhibitor than MDP, suggesting that a *N*-acetylglucosamine residue contributed to binding. An intact glycopeptide molecule was required because disaccharide and dipeptide fragments of GMDP were ineffective (Fig. 1, lanes 6, 7). The absence of inhibitory activity of LL-GMDP (Fig. 1, lane 5) indicated that the stereochemistry of an isoglutamine residue was also important. These results were in agreement with the specificity of GMDP-binding sites detected in permeabilized murine macrophages [3] as well as with structural requirements for biological activity of glucosaminylmuramyl peptides [2], suggesting potential functional implications of binding to p18, p32 and p34.

We used the protease protection assay to confirm that p18, p32 and p34 were located inside the cells rather than on the cell surface. The cells (murine peritoneal macrophages or WEHI-3 cells) were treated with three proteases with different specificities (trypsin, papain or pronase) and in all three cases, the GMDP-binding proteins remained intact, evidently due to their intracellular location (data not shown).

Upon centrifugation of the lysates of the cells (either peritoneal macrophages or WEHI-3) at $102\,000 \times g$ for 1 h, p18, p32 and p34 sedimented with the pellet indicating that these were not soluble cytoplasmic proteins. These proteins were solubilized, though when the pellets were treated with 2 M KCl in the case of murine peritoneal macrophages and by 2 M KCl or 0.01 M EDTA in the case of WEHI-3 cells. As was shown by SDS-PAGE, this procedure enabled us to separate GMDP-binding proteins from the bulk of cellular proteins.

We used the anion-exchange HPLC to purify the GMDP-binding proteins from the WEHI-3 EDTA extract. Elution with a NaCl gradient was employed. SDS-PAGE with subsequent Western blotting revealed that at a neutral pH, GMDP-binding proteins eluted at the end of the gradient, later than the majority of EDTA-solubilized proteins (Fig. 2).

In the case of macrophages, DEAE-chromatography could hardly be used because of the high salt content in KCl extract. Dialysis or diafiltration resulted in loss of p32 and p34, evidently due to sticking to the membrane. Therefore, macrophage subcellular fragments were first extracted with 0.01 M EDTA to partly remove contaminating proteins and then with 2 M KCl. The high pressure gel chromatography was employed to purify the GMDP-binding proteins from the KCl extract. The bulk of GMDP-binding activity eluted as a 130–

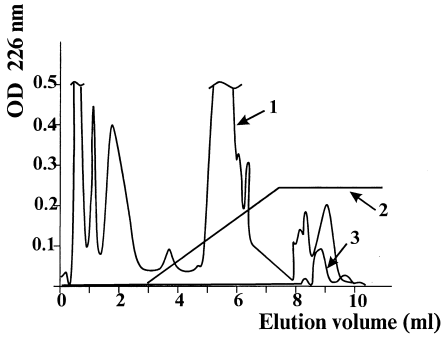

Fig. 2. Purification of GMDP-binding proteins from WEHI-3 cells by DEAE-HPLC. EDTA extract of WEHI-3 cells was subjected to HPLC on a TSK DEAE-5PW column. Proteins were eluted with a 0–0.5 M NaCl gradient. 1, Protein profile (OD_{226}); 2, NaCl gradient; 3, GMDP-binding activity.

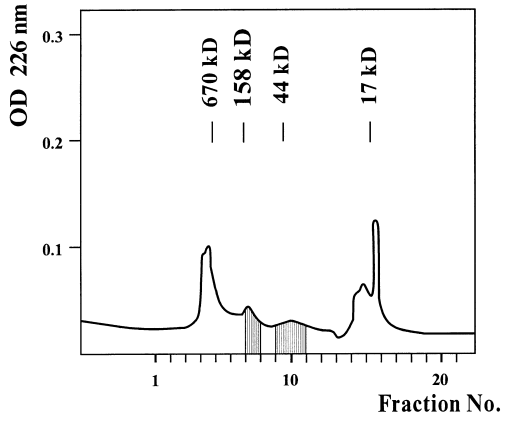

Fig. 3. Purification of GMDP-binding proteins from murine macrophages by DEAE-HPLC. Gel filtration of macrophage KCl extract was carried out on a G2000SW HPLC column in 20 mM Tris-HCl buffer, pH 7.6, containing 2 mM Na-EDTA and 0.5 M NaCl. Hatched areas, GMDP-binding activity.

AcSEAAPAAPAAAPPAEKAPAKKKAAKK
PAGVRR**KASGPPVSELIT**KAVAASKERS
GVSLAALKKA**LAAAGYDVEK**NNSRIKL
GLKSLVSKGILVQTKGTGASGSFKLNKK
AASGEAKPQAKKAGAAKAKKPAGAAK
KPKKATGAATPKKAAKKTPKKAKKPAA
AAVTKKVAKSPKKAKVTKPKKVKSASK
AVKPKAAKPKVAKAKKVAAKKK

Fig. 4. Amino acid sequence of murine histone H1A [22]. Peptides isolated from WEHI-3 cells are underlined.

140 kDa molecular mass protein (Fig. 3, fraction 7). According to SDS-PAGE carried out under non-reducing conditions, this peak consisted of equivalent amounts of p32 and p34, assuming a non-covalently linked oligomeric structure. The p18 eluted as a broad peak within the 35–65 kDa molecular mass interval (fractions 9, 10), indicating an oligomeric structure as well.

The GMDP-binding proteins from both macrophages and WEHI-3 cells were further purified by SDS-PAGE and blotted onto an Immobilon membrane for N-terminal sequencing. In all cases, the N-terminal amino acid turned out to be blocked. Therefore, to obtain sequence information after SDS-PAGE, in-gel trypsin digestion was performed followed by isolation of peptides by RP-HPLC. Sequencing of peptides obtained from p32 and p34, isolated from either cell type, matched sequence of histone H1 [22] (Fig. 4). The structure of histones H1 is highly conserved between species. Their calculated molecular mass is 21.5 kDa, but they were shown to migrate on SDS-PAGE gels as 32–34 kDa proteins due to high lysine content as was demonstrated, for instance, for the human H1b protein [23].

The purification of p18 was achieved by SDS-PAGE of the GMDP-binding fraction from DEAE-chromatography of WEHI-3 cell lysate. The sequencing of two peptides obtained after in-gel trypsin digestion matched the sequences of two fragments corresponding to amino acids 42–50 and 74–78 of the histone H3.1 [24].

Thus, GMDP-binding proteins, isolated by us, turned out to be histones. Histones are known to be strongly basic proteins which can bind acidic substances non-specifically due to ionic interactions. That this was not the case for GMDP was proved by the inability of LL-GMDP, which had the same pI value as GMDP, to inhibit GMDP interaction with histones. Besides, the variation of the pH of buffer within the 4.5–7.5 range did not affect the binding of (GMDP-Lys)-PAA-(Bi) to receptor proteins confirming this conclusion (data not shown).

Histones are located predominantly in the cell nucleus, though H1 protein was reported to be found on the cell surface and cell cytoplasm as well [25]. Sorace and Johnson [26] have shown that monoclonal anti-leukemic antibody capable of binding to a wide variety of cell lines was specific to histone H1. Recently, Brix et al. [27] reported that extracellularly occurring histone H1 mediated the binding of thyroglobulin to the cell surface of mouse macrophages. Nevertheless, our data [4] as well as data from other laboratory [5,6] favor the intracellular location of functional muramyl peptide-binding sites and, hence, possible involvement of nuclear histones in GMDP activity.

GMDP is not unique among peptide immunomodulators in its ability to bind to histones. Previously, prothymosin-a1, the inducer of T-cell proliferation, was found by Papamarcaki et al. [28] to interact with histone H1. Besides, a number of polypeptide hormones were shown to have binding sites in the cell nucleus, in particular, epidermal growth factor, nerve growth factor, platelet-derived growth factor, etc. [29,30]. Their binding to chromatin was assumed to cause structural alterations resulting in an increased DNA accessibility to transcription factors and enzymes and leading to gene activation [31–33]. Therefore, binding of GMDP to H1 is of particular interest because it is thought to be primarily responsible for the higher order organization of nucleosomes into the thick, transcriptionally inert chromatin fiber [34]. It might be assumed that GMDPs, upon interaction with histones, can loosen their binding to DNA, making gene regulatory sequences more accessible to transcription enzymes and resulting in gene expression. The gene-inducing activity of muramyl peptides, in particular their effect on cytokine genes expression, was reported earlier [35,36]. The key question remains if muramyl peptides can indeed enter the cell nucleus or not. Richerson et al., using fluorescence microscopy, demonstrated the uptake of FITC-labelled MDP-Lys by and accumulation inside rabbit alveolar macrophages [37]. They observed both diffuse and speckled cytoplasmic but not nuclear fluorescence. Nevertheless, these experiments do not exclude that muramyl peptides can penetrate the nuclear membrane and bind to chromatin in vivo because non-physiological conditions (4°C) and a relatively short incubation time (1 h) were used. Besides, bright cytoplasmic fluorescence could hinder weak nuclear fluorescence. Our preliminary data obtained by using the laser scanning confocal microscopy point out that this was the case. It was shown that at 37°C after 3 h (but not 1 h) incubation, GMDP-Lys-FITC is found within the macrophage nucleus.

The data obtained show that histones H1 and H3 can specifically interact with muramyl peptides. It is still to be proved that this interaction takes place in vivo, but in any case, it is evident that there are a number of target molecules for muramyl peptides in mammalian cells, among which are serotonin receptors [8] and a few other proteins [7,11]. Thus, it can be postulated that the observed variety of biological effects of muramyl peptides results from their interaction with a number of cellular proteins.

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