The Molecular Mechanism of Muramyl Peptides' Biological Activity

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Muramyl peptides (MPs) are known to influence greatly the immune response [1]. They are active as adjuvants, induce nonspecific resistance to certain viral and bacterial infections, and in some cases are active against various tumors. Many studies attempting to elucidate the mechanism of their biological activity have been carried out. However, the molecular basis of MP's effects on the immune system is still unclear.

The immune network includes at least three circuits: idiotype–antiidiotype interactions [2], the cytokine network [3], and the regulation of receptor expression on immunocompetent cells. MPs are known to induce cytokine production, and so clearly interfere with the cytokine cascade [4]. They also influence idiotype network, as they increase production of immunoglobulin. Little if anything is known about their effect on the expression of cell membrane molecules [5]. We approached this subject by studying oligosaccharide-containing MPs synthesized in our institute by T. Andronova, E. Makarov, and V. Ivanov.

Macrophages are known to be the primary target for MPs [1]. As these cells function as antigen-presenting cells, we evaluated the effect of *N*-acetylglucosaminyl-β 1-4-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (GMDP, Fig. 1) and its analogs on major histocompatibility complex (MHC) class II antigen expression, as these glycoproteins are crucial for recognition of antigens by T-cells [6]. BALB/c mice peritoneal macrophages were used. The number of Ia-positive cells was estimated by flow cytometry after labelling cells with biotinylated anti-Ia monoclonal antibodies and fluorescein isothiocyanate (FITC)-labelled avidin.

To summarize briefly the results obtained [7], it was found that:

1) Incubation of macrophages with GMDP in vitro caused a dose-dependent increase in the Ia-positive cell number and fluorescence intensity. Ia expression peaked at 18 h and persisted for at least the next 30 h.
2) This effect was also observed in vivo upon injection of GMDP into the peritoneal cavity of mice.
3) Biologically active MPs, with few exceptions, also possessed Ia-inducing activity; nonactive compounds were always inactive in this assay.
4) MPs directly affected macrophage. This was evident from the increase in Ia-antigen expression on myelomonocytic leukemia cells (WEHI-3). The effect could not be attributed to intermediate formation of tumor necrosis factor α (TNF-α), the known Ia inducer, as we failed to find TNF-α in WEHI-3 culture medium. At present, the involvement of other interleukins cannot be excluded.
5) The observed effect was not species specific: not only murine peritoneal macrophages but human monocytes as well could be induced to express MHC class II (HLA-DR) antigens, though the magnitude of the effect was lower.

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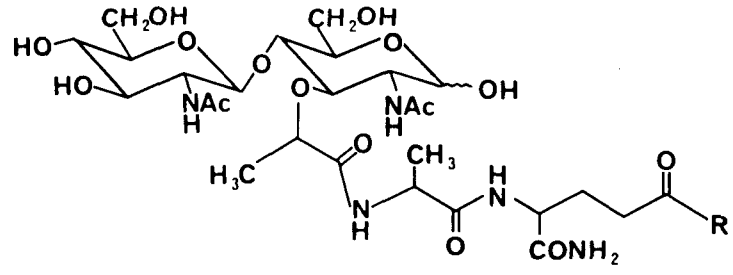

GMDP: R=OH

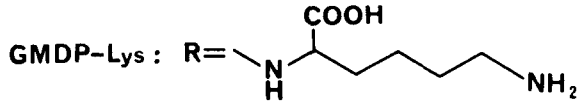

Fig. 1. *N*-Acetylglucosaminyl-β1-4-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (*GMDP*) and GMDP-Lys

The maximal HLA-DR expression was observed at 0.1–1 μg GMDP/ml.

6) Finally, besides MHC class II antigens, the expression of interleukin-2 (IL-2) receptors was induced by GMDP. Taking in account that IL-2 was shown to increase the cytotoxicity of macrophages against tumor targets, we assume that this mechanism might be involved in the antitumor activity of GMDP.

It was shown previously that MPs have no direct cytotoxic effect on tumor cells; rather, they kill tumor cells by activating the immune system [8]. We assumed that there might be another effect, namely an increase in expression of tumor-associated antigens and MHC antigens on tumor cells, resulting in their being better recognized by immunocompetent cells. Human lung adenocarcinoma cells (RL-4) and colon adenocarcinoma cells (WiDr) were used as tumor targets. These cells are known to express various levels of carcinoembrionic antigen (CEA) as tumor-associated marker. The expression of CEA and RL-4 cells was monitored by labelling the cells with FITC-anti-CEA monoclonal antibodies followed by flow cytometric enumeration of labelled cells.

RL-4 cells cultured without stimulant expressed only minute amounts of CEA. Incubation of RL-4 cells (3×10^5) with GMDP resulted in a dose-dependent increase in CEA expression with maximal expression at 10 μg/ml (Fig. 2). The number of CEA-positive cells peaked at 18–24 h. An additive effect was observed upon combined treatment of RL-4 cells with GMDP and interferon-γ (IFN-γ).

The treatment of RL-4 cells with GMDP also resulted in increased expression of HLA-DR antigens.

For WiDr cells an increase in CEA-expression after 48 h incubation was observed as well. In this case, flow cytometry could not be used due to clumping of cells. The CEA expression by WiDr cells was monitored by cell enzyme-linked immunosorbent assay (ELISA).

Whether the above-mentioned effects have functional implications regarding the recognition of tumor cells by the immune system remains to be studied.

Another goal of our study was to identify the MP-binding molecules on responding cells, as the existence and location of specific cellular MP receptors were controversial [9, 10, 11]. To address this question we used two approaches.

The first approach was based on direct staining of MP-binding cells with FITC-labelled GMDP-Lys: the FITC group was attached to the ε-amino group of lysine (see Fig. 1). Fluorescence-activated cell sorting (FACS) analysis of intact murine peritoneal macrophages and WEHI-3 cells showed no specific binding, but when the cell membrane was first fixed with paraformaldehyde and permeabilized with β-octylglucoside and then treated with GMDP-Lys-FITC,

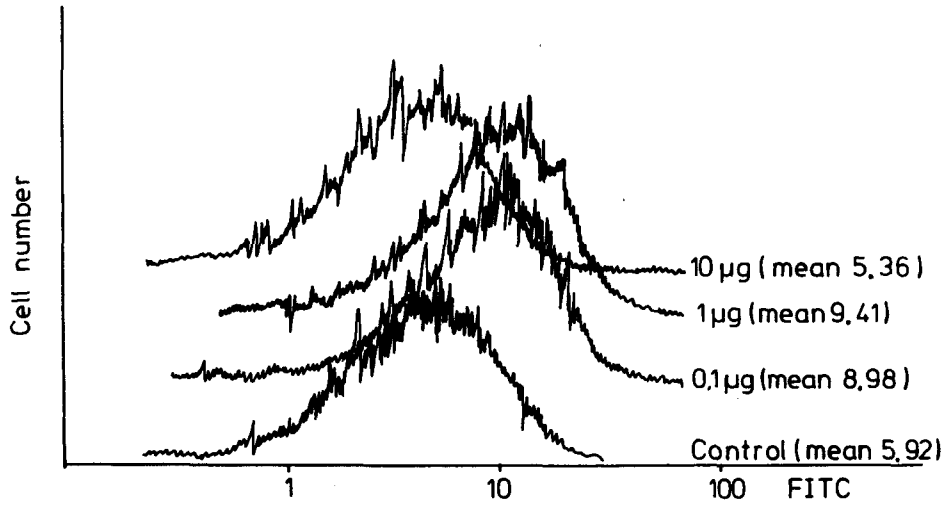

Fig. 2. Effect of incubation with GMDP on CEA expression by RL-4 cells

binding to macrophages and WEHI-3 cells was observed. This binding could be inhibited effectively by GMDP or GMDP-Lys. In contrast, the tripeptide Ala-Gln-Lys was rather ineffective as an inhibitor.

Thus, the GMDP-binding molecule seems to be located inside macrophages. The number of specific binding sites on WEHI-3 cells estimated by comparison with fluorescein-labelled beads of known molecular composition was in the range of 6×10^4, though the total number of binding sites was much higher (2.2×10^5). These values are lower than those obtained for rabbit bronchoalveolar lavage cells [12].

The second approach was based on evaluation of fluorescence polarization of GMDP-Lys-FITC upon binding to cells. Similarly to FACS analysis, only for permeabilized cells was specific binding observed, detected by a change in fluorescence polarization. The ability of GMDP and GMDP-Lys to compete with fluorescent congeners for binding sites suggested specificity of binding. The number of specific binding sites per cell calculated from these data was in the range of $4-5 \times 10^4$ and agreed reasonably well with data obtained by FACS analysis. The Scatchard plot suggested the presence of two populations of binding sites with K_d 2×10^{-8} M and 5×10^{-7} M (Fig. 3).

The above findings are consistent with intracellular MP-binding molecules. Thus, to display immunomodulatory activity MP must be internalized by macrophage. The binding of GMDP to a receptor molecule results in initiation of biosynthesis or enhanced biosynthesis of certain proteins, including cytokines and cell membrane molecules. The changes in

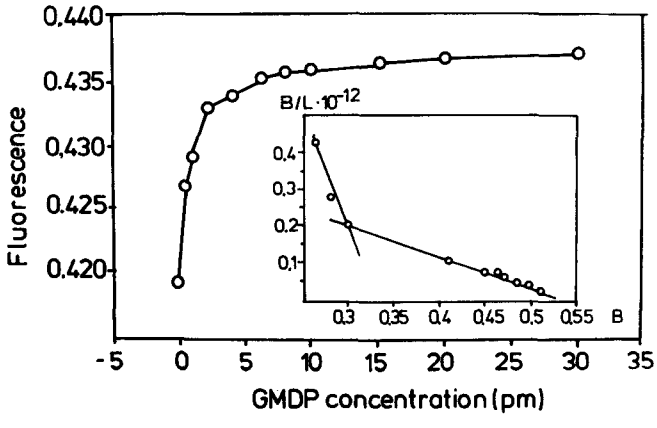

Fig. 3. Binding of GMDP

surface antigen expression influence the magnitude of the immune response.

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