Volume 220, number 1, 93-97 FEB 04967 August 1987

A novel muramyl peptide derivative stimulates tumoricidal activity of macrophages and antibody production by B cells

Jean-Pierre Tenu, Arlette Adam, Vongthip Souvannavong, Gillian Barratt, Alexandre Yapo, Jean-François Petit, Michel Level*, Martin Clemance+ and Kenneth Douglas+

*Institut de Biochimie, Bât.432, Université Paris-Sud, 91405 Orsay, *Institut Choay, 10 rue Morel, 92120 Montrouge, France* and *+ Department of Chemistry, University of Essex, Colchester, CO4 3SQ, England*

Received 22 May 1987

A novel analog of MDP, the 3'-iodo-4'-azido-L-phenylalanine methyl ester derivative of *N*-acetyl-L-alanyl-D-isoglutamine, has been prepared. This compound is capable of activating macrophages to the tumoricidal state and increasing the specific immune response of B cells. It thus appears to exhibit similar biological activities to MDP. Moreover, this compound is of potential interest for receptor photolabelling studies.

Muramyl peptide; Adjuvant; Antibody production; Macrophage activation; (B cell)

1. INTRODUCTION

A water-in-oil emulsion of killed mycobacteria has been known since the forties to exhibit an immunoadjuvant action. Indeed, 'Freund's complete adjuvant', as this preparation is known, is used commonly by immunologists to stimulate antibody production and induce cellular immunity. It was not until the early seventies, however, that the minimal chemical structure necessary to reproduce the effects of Freund's adjuvant became defined as *N*-acetyl-muramyl-L-alanyl-D-isoglutamine **(1)**, (MDP or muramyl dipeptide) [1].

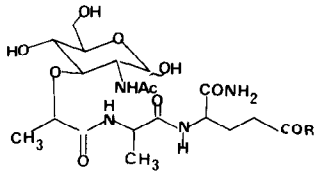
Correspondence address: J.-P. Tenu, Institut de Biochimie, Bât. 432, Université Paris-Sud, 91405 Orsay, France

Abbreviations: MDP, muramyl dipeptide; AI-MDP, *N*-acetyl-muramyl-L-alanyl-D-isoglutaminyl-(3'-iodo-4'-azido)-L-phenylalanine methyl ester; MEM, minimal essential medium; FCS, fetal calf serum; PBS, phosphate buffered saline; FRM, macrophage replacing factor; SRBC, sheep red blood cell; PFC, plaque forming cells; LPS, lipopolysaccharide; MLC, mixed lymphocyte culture

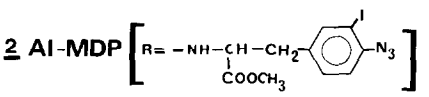
MDP has a multiplicity of biological effects (review [2]) including potentiation of specific immune responses, as well as an increase in natural resistance to microbial infections and tumor development and a more recently described sleep-promoting effect [3].

More specifically, MDP has been shown to interact directly with, and elicit biological responses from, macrophages and B cells in vitro. For instance, with macrophages, MDP enhances their tumoricidal action [4]. There has been considerable discussion recently of the possible role(s) and location(s) of specific muramyl peptide binding receptors on activated macrophages and of whether such cell-surface receptors exist [5–7]. It has been suggested that under some circumstances a common set of receptors are shared between muramyl peptide and serotonin [8]. With respect to B cells, MDP can increase their primary in vitro antibody response in appropriate conditions and it appears that MDP acts directly on B cells, in a late stage of their differentiation to antibody-producing cells [9]. To address in molecular detail questions such as the location and identity of MDP receptor sites (and whether there are several classes of these), we have prepared **2** (fig.1). This

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50



1 MDP [R = OH]



2 AI-MDP [R= -NH-CH(COOCH3)-CH2- (3-iodo-4-azidophenyl)]

Fig.1. Structure of MDP and AI-MDP.

derivative, which is a photolabile analog of **1**, was designed based on the known structure-activity relationships in the MDP family. This derivative, the 3′-iodo-4′-azido-L-phenylalanine methyl ester analog of MDP (AI-MDP), incorporates an aryl azide moiety (from which a highly reactive nitrene can be photogenerated) which provides the potential to photolabel MDP receptors. We report here the biological activities of **2** towards macrophages and B cells.

2. MATERIALS AND METHODS

2.1. *Synthesis*

Derivative **2** was synthesised by isobutylchloroformate-mediated coupling of MDP with 3′-iodo-4′-azidophenylalanine methyl ester. The product was purified by HPLC using an IBM-controlled HPLC system from Gilson or a Waters 600 instrument and reversed-phase isocratic chromatography on a Zorbax C_8 column (Dupont Instruments) (using MeOH/H_2O, 60:40 as a solvent system).

Homogeneity of the sample was tested not only by HPLC but also for a range of TLC solvent systems. An independent synthesis of **2** by an alternative route gave a product with identical HPLC characteristics. The structure and identity of **2** were confirmed by NMR and elemental analysis. Full details of the synthetic and purification procedures used will be published elsewhere. For biological studies, the freeze-dried compound AI-MDP was dissolved in apyrogenic, deionised water to provide stock solutions.

2.2. *Animals*

Female Wistar Albino Glaxo (WAG) rats, 8–10 weeks old, were obtained from CSEAL (Orléans, France) as were 7–12-week-old female BDF1 (C57Bl/6xDBA/2), C57Bl/6 and DBA/2 mice.

2.3. *Adjuvant, antigen and medium*

MDP and derivatives were provided by Choay Laboratories (Montrouge, France). Sheep erythrocytes were obtained from a selected single donor and kindly provided by the CNRZ (INRA, Brouessy, France). We used RPMI-1640 (Gibco) medium supplemented with 25 mM Hepes, L-glutamine, 8% heat-inactivated FCS (Gibco), 50 μM 2-mercaptoethanol and standard antibiotics.

2.4. *Preparation of FRM*

BDF1 resident peritoneal cells were cultured at 1×10^6/ml for 90 min and the non-adherent cells removed by washing. A further overnight incubation was carried out to prepare the supernatant of adherent cells which was concentrated 10-fold with Immersible-CX (Millipore) and then chromatographed on Ultrogel AcA54 (IBF). FRM eluted in the fraction of apparent molecular mass 35 kDa.

2.5. *Production of MLC supernatants*

Allogeneic culture supernatants were obtained by mixing whole spleen cells from normal DBA/2 and C57Bl/6 mice at 10^7 cells/ml for 24 h. The MLC supernatant contained the complete set of helper factors capable of restoring the immune responsiveness of B cells [9]: it was used at suboptimal doses.

2.6. *Cell preparations*

Macrophages were removed by passage of BDF1 splenocytes through Sephadex G-10 columns: this preparation is referred to hereafter as G-10-treated cells. To obtain B cells which were depleted of both macrophages and T cells, the G-10-treated cells (10^7 cells/ml) were incubated with a monoclonal F7D5 anti-Thy-1.2 antibody (1/500, Olac, Bicester, England) for 30 min at 37°C and then for 45 min at 37°C with complement (C, 1/10).

2.7. *Culture assays*

10^6 splenocytes, G-10 passed cells or purified B cells were cultured in flat bottom wells with 10^6

SRBC (total volume 200 μl). The IgM anti-SRBC PFC response was determined on day 5. The PFC values given are the means of quadruplicate cultures.

2.8. *Preparation of alveolar macrophages*

Macrophages were lavaged from the lungs of rats with normal saline as described [10]. The resulting cell suspension in MEM containing 5% FCS (endotoxin-free) was supplemented with antibiotics. Cells (10^5 or 2×10^5 in 0.25 ml) were introduced into the wells of 96-well plates (Falcon Plastics, Oxnard, CA). Macrophages were allowed to adhere for 3–4 h, and then washed twice with PBS.

2.9. *Tumor cells*

The P77 syngeneic tumor line used was cloned from a lung fibrohistocytoma radioinduced in WAG rats [11,12].

2.10. *Cytotoxicity assay*

This was a modification of a previously described method [13] in which the cumulative incorporation of [^{3}H]thymidine into tumor cell DNA over a 20 h period was measured. Tumor target cells (2.5×10^4, P77) were added to each well in MEM containing 10% FCS, antibiotics, [^{3}H]thymidine (28 Ci/mmol, CEA, Saclay, France) mixed with unlabelled thymidine to give a final thymidine concentration of 1.2 μM and a specific activity of 1 Ci/mmol. The medium was supplemented with 20 ng/ml of LPS (Difco) unless otherwise stated. After 20 h incubation at 37°C with 5% CO_2, the radioactivity incorporated was collected on glass fiber filters in a cell harvester (Skatron, Norway) and counted in a β-scintillation spectrometer (LKB).

Tumor cells were also cultivated in the absence of macrophages under similar conditions. The validity of this assay, in which incorporation of [^{3}H]thymidine is directly proportional to the increase in the number of viable tumor cells, has been demonstrated [14].

3. RESULTS AND DISCUSSION

The new derivative of MDP, AI-MDP (**2**), was synthesised by an unequivocal route (see section 2). A prerequisite for the use of derivative **2** as analog of MDP is to demonstrate the existence and nature of its biological activity relative to that of MDP. In this work, studies are reported to exemplify the activity of AI-MDP (**2**) in the immune system with both nonspecific resistance (in macrophages) and specific immunity (in B cells). We shall deal first with the results of macrophage studies.

3.1. *Activation of macrophages towards tumor cytotoxicity by MDP and AI-MDP*

Unlike mouse peritoneal macrophages, rat alveolar macrophages respond both well and reproducibly to MDP, and consequently were used in this work [4]. Rat alveolar macrophages incubated for 24 h with MDP or AI-MDP were able

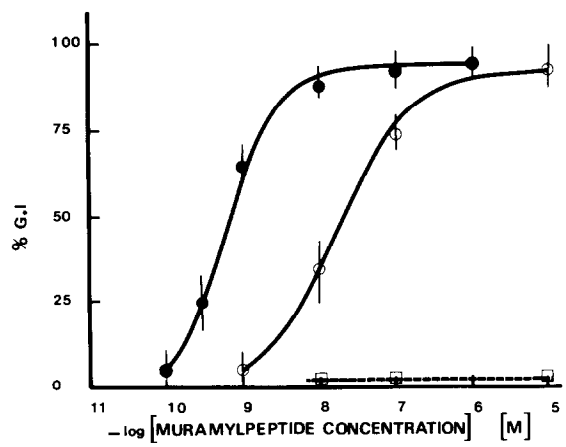


Fig.2. Rat alveolar macrophages were incubated at 8×10^5/ml in 96-well plates with muramyl peptides at various concentrations in MEM medium supplemented with 5% FCS (LPS-free) and 20 ng/ml added LPS. After 24 h, the macrophages were washed and 10^5 P77 target cells were added in the presence of [^{3}H]thymidine for 20 h. Incorporation of label into tumor cell DNA was measured as described in section 2 and the results are expressed as percentage growth inhibition (% GI):

$$\% \text{ GI} = 100 \left(1 - \frac{X}{R}\right)$$

where X is the incorporation into the DNA of tumor cells cultured with treated macrophages and R the incorporation into tumor cells cocultured with macrophages exposed to medium only. The latter value was always 80% or more of the incorporation into tumor cells cultured alone, thus the macrophages were not intrinsically cytotoxic. % GI values given are means of results from quadruplicate cultures. MDP (○); AI-MDP (●); MDP(D,D) (□).

to inhibit the growth of syngeneic P77 tumor cells at an effector:target ratio of 8, provided that a suboptimal amount of LPS (20 ng/ml), which is inactive per se, was present during induction of cytotoxicity, as reported for MDP [10]. Typical results are shown in fig.2. Similar results were obtained at an effector:target ratio of 4 except that the maximum percentage of growth inhibition reached in the presence of AI-MDP was 78% (cf. 94% obtained for fig.2). Clearly, under these conditions AI-MDP is effective in stimulating tumor growth inhibition by the macrophages.

From fig.2 it can be seen that the activating effect of muramyl peptides is dose-dependent, the half-effective concentration being in the range 10^{-8}–10^{-7} M for MDP and in the range 10^{-10}–5×10^{-9} M for AI-MDP, depending on the particular macrophage preparation used. Thus, it appears that AI-MDP is one to two orders of magnitude more potent than MDP itself in this bioassay. In these studies MDP-D,D (the stereoisomer of MDP with D- in place of L-alanine) was completely incapable of activating rat alveolar macrophages for tumor cytotoxicity. Serotonin (10^{-7} to 10^{-5} M), methylsergide and ketanserin (serotonin antagonists at 10^{-6} to 2.5×10^{-5} M) were also completely inactive. This behaviour contrasts with recent results [8] describing serotonin as active in inducing $O_2^{\cdot-}/H_2O_2$ generation by macrophages. On detailed analysis these results are not conflicting if we take into account the fact that $O_2^{\cdot-}/H_2O_2$ generation by macrophages is a good marker of the primed state of macrophages, but not of the activated antitumor state [15].

3.2. *Specific antibody response of B cells stimulated by MDP and AI-MDP*

The effects of MDP and AI-MDP on the specific in vitro immune response to SRBC are summarized in fig.3. The results of fig.3A show that the increase of the PFC response, the so-called adjuvant effect, is dose-dependent and increases up to a concentration of about 10^{-6} M. The lower values obtained at higher concentrations are caused by the stimulation of suppressor cells which has been shown to occur at high concentrations of MDP [16]. The optimum increase of the PFC response induced by AI-MDP is obtained at a much lower concentration. On the basis of fig.3A, AI-MDP is at least one order of magnitude stronger at increas-

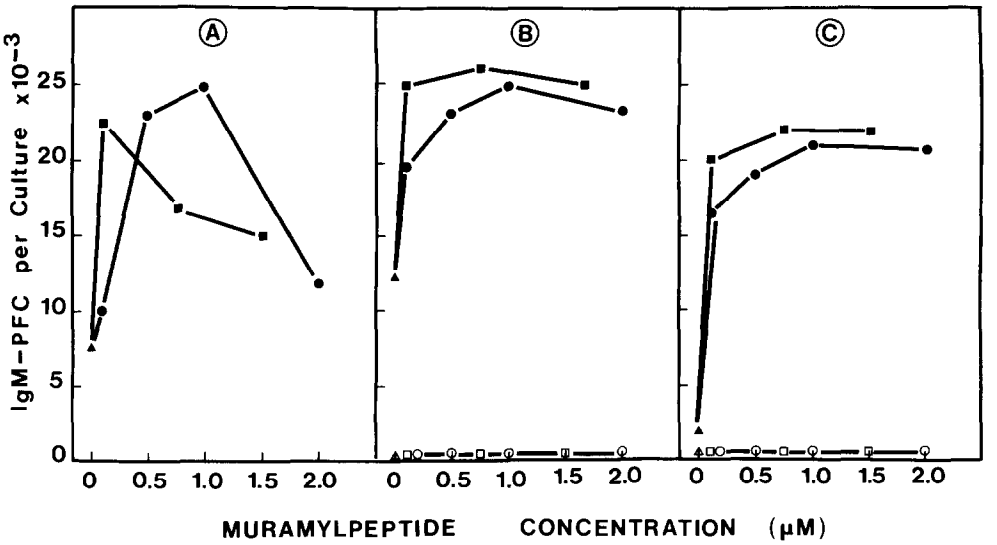


Fig.3. Effect of various doses of MDP and AI-MDP on the in vitro immune response to SRBC as measured by the number of PFC at day 5. Spleen cells (A), macrophage-depleted spleen cells (B) and B cells (C) were cultured at 10^6 cells/200 μl well with 5×10^5 SRBC, in the absence or presence of either FRM (B) or MLC supernatants (C). Values represent the mean of results from quadruplicate wells. MDP (○,●) or AI-MDP (□, ■) were added to cultures in the absence (○, □) or presence (●, ■) of either FRM (B) or MLC supernatants (C). Control cultures for (A) whole spleen cells (▲); (B) G10-passed cells with (▲) or without (△) FRM; (C) B cells with (▲) or without (△) MLC supernatant.

ing antibody production in spleen cells than MDP itself. Moreover, the dose-response curves for both MDP and AI-MDP are qualitatively similar, possibly indicating parallel behaviour.

Fig.3B summarises the results obtained with MDP and AI-MDP with respect to the specific immune response to SRBC of macrophage-depleted spleen cells (i.e. G10-treated cells, see section 2) and of B cells (i.e. spleen cells stripped of their macrophage and T cell components). The G10-treated cells do not respond to SRBC (with or without muramyl peptide adjuvant) unless supplied with a monokine, in this case FRM which is produced spontaneously by unstimulated macrophages [17]. Relative to FRM, MDP and AI-MDP increase the PFC value about 2-fold but there appears to be no significant difference in effectivity of MDP and AI-MDP in the stimulation of macrophage-depleted cells.

In the case of purified B cells (fig.3B) the immune response is not evident unless interleukins are present (here provided by MLC supernatants), even with MDP and AI-MDP present at concentrations of 10^{-7} to 5×10^{-6} M. However, in the simultaneous presence of suboptimal levels of interleukins and either MDP or AI-MDP, a highly synergistic increase of antibody production is observed (an increase of about 10-fold).

In summary, AI-MDP is active as an analog of MDP both towards macrophages and towards spleen cells, especially B lymphocytes. Indeed, not only does AI-MDP exhibit apparently parallel behaviour with MDP in the systems tested to date, but it is also one to two orders of magnitude more potent than MDP. In view of the presence of the arylazide group [18] (a photosensitive precursor useful in photoaffinity labelling), and the iodo substituent (a ^{125}I derivative will allow low levels of receptors to be detected and studied), AI-MDP presents itself as an important potential tool in studies of MDP receptors. Such work is now under way in our laboratories.

ACKNOWLEDGEMENTS

We are grateful to Professor E. Lederer for constant interest in this work, and wish to acknowledge the Wellcome Trust for support (M.C.) and to NATO for travel funds allowing the reality of a bench-level collaboration in this difficult area. We thank Dr D. Vergé for the generous gift of ketanserin and methylsergide.

REFERENCES

[1] Ellouz, F., Adam, A., Ciorbaru, R. and Lederer, E. (1974) Biochem. Biophys. Res. Commun. 59, 1317–1325.
[2] Adam, A. and Lederer, E. (1984) Med. Res. Rev. 4, 111–152.
[3] Krueger, J.M., Pappenheimer, J.R. and Karnovsky, M.L. (1982) J. Biol. Chem. 257, 1664–1669.
[4] Sone, S. and Fidler, I.J. (1981) Cell. Immunol. 57, 42–50.
[5] Tenu, J.-P., Roche, A.C., Yapo, A., Kieda, C., Monsigny, M. and Petit, J.F. (1982) Biol. Cell. 44, 157–164.
[6] Fogler, W.E. and Fidler, I.J. (1986) J. Immunol. 136, 2311–2317.
[7] Silverman, D.H.S., Krueger, J.M. and Karnovsky, M.L. (1986) J. Immunol. 136, 2195–2201.
[8] Silverman, D.H.S., Wu, H. and Karnovsky, M.L. (1985) Biochem. Biophys. Res. Commun. 131, 1160–1167.
[9] Souvannavong, V. and Adam, A. (1984) Biochem. Biophys. Res. Commun. 125, 431–439.
[10] Barratt, G.M., Tenu, J.-P., Nolibé, D. and Petit, J.F. (1986) Proceedings of the 2nd International Workshop on the Biological Properties of Peptidoglycan, Munich, May 1985 (Seidl, P.H. and Schleifer, K.H. eds) pp.249–254, Walter de Gruyter, Berlin.
[11] Nolibé, D., Berek, E., Masse, R. and Lafuma, J. (1981) Biomedecine 35, 230–234.
[12] Nolibé, D., Aumaitre, E. and Thang, M.N. (1985) Cancer Res. 45, 4774–4778.
[13] Williams, R.M., Germain, R.N. and Benacerraf, B. (1975) J. Natl. Cancer Inst. 54, 697–708.
[14] Lepoivre, M., Tenu, J.-P., Lemaire, G. and Petit, J.F. (1982) J. Immunol. 129, 860–866.
[15] Grand-Perret, T., Lepoivre, M., Petit, J.F. and Lemaire, G. (1986) Eur. J. Immunol. 16, 332–338.
[16] Souvannavong, V. and Adam, A. (1980) Eur. J. Immunol. 10, 654–656.
[17] Souvannavong, V., Rimsky, L. and Adam, A. (1983) Biochem. Biophys. Res. Commun. 114, 721–728.
[18] Bayley, H. and Knowles, J.R. (1977) Methods Enzymol. 46, 69–114.