Vol. 96, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1980 Pages 915-923

DISSOCIATION OF IMMUNOSTIMULANT ACTIVITIES OF MURAMYL DIPEPTIDE (MDP) BY LINKING AMINO-ACIDS OR PEPTIDES TO THE GLUTAMINYL RESIDUE

F. Audibert[+Φ], M. Parant[+], C. Damais[+], P. Lefrancier[°],

M. Derrien[°], J. Choay[°] and L. Chedid[+]

[+] Institut Pasteur, Groupe de Recherche No.31 du C.N.R.S., Immunothérapie Expérimentale, 28, rue du Dr. Roux, 75724 Paris cedex 15, France.
[Φ] INSERM, Paris.
[°] Institut Choay, Paris.

Received July 18,1980

N-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide or MDP) is the minimal structure required for substituting mycobacteria in Freund's complete adjuvant. It is an adjuvant when injected in saline, protects mice non-specifically against infection, and enhances thymidine incorporation of lymphocytes. In this report it is shown that the linking of L-Lys, L-Ala-D-Ala, L-Lys-D-Ala or L-Lys-L-Ala to MDP permits the dissociation of anti-infectious activity from adjuvant activity. The optical configuration of the added residues plays the major role in this dissociation. It can be noted that the muramyl tetrapeptide MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala mimicking the natural structure is devoid of anti-infectious activity.

The minimal structure which can substitute for mycobacteria in Freund's complete adjuvant was shown in 1974 to be synthetic MDP, or *N*-acetylmuramyl-L-alanyl-D-isoglutamine (1). It represents part of the constitutive, repetitive subunit of some bacterial peptidoglycans since this subunit is *N*-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine (2). The adjuvant activity of MDP was demonstrated first when injected in a water-in-oil emulsion (1, 3, 4), then when administered in saline (5), and even when given orally (6). Furthermore, it has the capacity to protect mice non-specifically against lethal infection (8) and to enhance thymidine uptake by mouse spleen cells (9). To iden

Abbrevations used: MurNAc : N-acetyl-muramic acid,
Z : benzyloxycarbonyl.

0006-291X/80/180915-09$01.00/0

tify the relevant chemical structures for various immunostimulatory effects, certain modifications were made which led to the findings that the two carboxyl groups of the glutamic acid residue and the stereochemistry of the peptide moiety are essential (3, 6, 7, 10, 11), in particular, neither MurNAc-D-Ala-D-isoGln, MurNAc-L-Ala-L-isoGln, nor MurNAc-D-Ala-L-isoGln were immunostimulatory. As indicated above in natural structures, the muramyl dipeptide derivative is linked by the γ carboxyl residue of D-isoGln to L-Lys and D-Ala, the corresponding synthetic tetrapeptide MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala has been shown adjuvant-active in oil (3, 10, 12), and in saline (10) but devoid of protective activity against infection (8).

This paper describes the influence of linking L-Lys, D-Ala, L-Ala, L-Lys-D-Ala and L-Lys-L-Ala to the isoglutaminyl group of MDP on various immunostimulatory properties. Previously described correlations can be found between the abilities of glycopeptides to act as an adjuvant *in vivo* or *in vitro* and to stimulate thymidine incorporation by mouse spleen cells (13). In addition the enhancement of resistance to infection can be differentiated according to the stereochemical structure of the amino-acid residues linked to MDP.

MATERIALS AND METHODS

Synthetic compounds. The following compounds have been used:

Mur-NAc-L-Ala-D-Glu-NH_2	: MDP	1
Mur-NAc-L-Ala-D-Glu(L-Ala)-NH_2	: MDP-L-Ala	2
Mur-NAc-L-Ala-D-Glu(D-Ala)-NH_2	: MDP-D-Ala	3
Mur-NAc-L-Ala-D-Glu(L-Lys)-NH_2	: MDP-L-Lys	4
Mur-NAc-L-Ala-D-Glu(L-Lys-NH_2)NH_2	: MDP-L-Lys-NH_2	5
Mur-NAc-L-Ala-D-Glu(L-Lys-L-Ala)-NH_2	: MDP-L-Lys-L-Ala	6
Mur-NAc-L-Ala-D-Glu(L-Lys-D-Ala)-NH_2	: MDP-L-Lys-D-Ala	7

The synthesis of some analogs of MDP has been previously described: 1, 7 (15), 2, 3, 4, 6 (16). The preparation of 5 is described below:

Optical rotation was measured with a Perkin Elmer 141-polarimeter. Elemental analysis was performed with a Perkin Elmer 240 Elemental Analyser after drying the sample for 20 hr at various temperature, 0.1 Torr.

5 MurNAc-L-Ala-D-Glu(L-Lys-NH_2)-NH_2, acetate. MurNAc-L-Ala-D-Glu-NH_2 (993 mg) were dissolved in 7 ml of dimethyl-formamide. To this solution were added 324 mg of N-hydroxybenzotriazole and 847 mg of N-ethyl-N'-(3-dimethylamino-propyl)-carbodiimide, hydrochloride. After 1 hr at room temperature 315 mg of Lys(Z)-NH_2 HCl was dissolved in 2 ml of dimethylformamide containing 0.22 ml of N-methylmorpholine. The reaction was performed for 72 hr, then the solvent was evaporated in vacuo. The ensuing residue was purified by chromatography first on a column of AG-50-W-X4 (15 x 1 cm), eluted with dimethylformamide/0.1N acetic acid (1:3) then on a column of AG-1-X-2 (15 x 1 cm), eluted with dimethylformamide/2.10^{-3}N acetic acid (1:3). The pure product was obtained after silica gel chromatography (ready-made column type B, Merck) the elution solvent being ethyl acetate/pyridine/acetic acid/water (5.5 : 2 : 0.6 : 1) : 720 mg $[\alpha]_D^{20}$ = + 25.7° (glacial acetic acid).

This compound was hydrogenated for 2 hr in 20 ml of glacial acetic acid in the presence of palladium 5 % on charcoal. The reaction mixture was filtered and concentrated in vacuo. The product was obtained as lyophilized powder :

560 mg $[\alpha]_D^{20}$ = + 24.1° (glacial acetic acid).

Anal. Calc. for $C_{27}H_{49}N_7O_{13}$, 0.25 CH_3COOH (694.76) %.
C 47.54; H 7.25; N 14.11; Found C 47.41; H 7.44; N 14.11.

Animals. Swiss female mice (Centre National de la Recherche Scientifique, Orléans, France) and male Hartley guinea pigs (Coblanbel, Roger Bellon, France) were used to determine the adjuvant activity.

Infection experiments were performed in 5 to 6-week-old Swiss mice (Iffa Credo, France). For *in vitro* assays of thymidine uptake splenocytes from DBA/2 mice (C.N.R.S., Orléans, France) were used.

Adjuvant activity. It was determined according to methods previously reported (5).

a) Guinea pigs (6 per group) received in both hind footpads 0.5 mg of ovalbumin (5X crystallized, Miles Laboratories) in 0.1 ml of Freund's incomplete adjuvant (FIA) emulsion. Delayed type hypersensitivity was evaluated by skin testing animals on day 18. After bleeding on day 21, antibodies to ovalbumin were determined by quantitative precipitation (Folin's method).

b) Swiss mice (groups of 8) received subcutaneously, in 0.5 ml of saline, 0.5 mg of bovine serum albumin (BSA, fraction V, Miles Laboratories). Thirty days later they received 0.1 mg of antigen by the same route. Experimental groups received MDP or analogs with the first administration of antigen. Anti-BSA titers were measured by passive hemagglutination of antigen-coated sheep red blood cells with sera either pooled on day 4 or collected separately on day 36.

Infectious challenge. Mice (8 per group) received synthetic glycopeptides by the intravenous route, the time being indicated in the text. *Klebsiella pneumoniae* strain of capsular type 2 was the same as in previous work (8). The *P*-values were obtained by using the adjusted chi-square method (14).

Cell cultures. Mouse spleen cells (1.5 x 10^6/ml) were suspended in RPMI 1640 medium (Flow Labs., Irvine, Scotland) containing antibiotics, 1 % glutamine, and supplemented with 2-mercapto-

ethanol (2-ME) at a final concentration of 5×10^{-5}M, as previously described. Evaluation of ^{3}H-thymidine uptake was reported in detail elsewhere (13).

RESULTS

Adjuvant activity when injected into guinea pigs as a water-in-oil emulsion. The adjuvant activity was tested in guinea pigs as described in Methods. The results presented in Table 1 show that there is no significant difference in adjuvanticity between MDP and the other compounds. Delayed type hypersensitivity was obtained as well as an enhancement of the humoral response. Data

Table 1

Adjuvant activity when injected in a water-in-oil emulsion to guinea pigs

Treatment[a]	Skin reaction mm of induration[b]	Antibody response[c]
Controls	0	8.68 ± 0.48
MDP	13 ± 3	12.25 ± 0.90
MDP-L-Ala	10 ± 2	12.46 ± 0.59
MDP-D-Ala	8 ± 2	12.09 ± 0.65
MDP-L-Lys	11 ± 2	12.04 ± 0.53
MDP-L-Lys-NH_2	14 ± 2	12.63 ± 0.87
MDP-L-Lys-L-Ala	10 ± 1.5	13.04 ± 0.47
MDP-L-Lys-D-Ala	11.5 ± 3	12.25 ± 0.90

[a] Guinea pigs (6 per group) received in each hind footpad 0.1 ml of emulsion (FIA/saline) containing 0.5 mg of ovalbumin with or without the various analogs.

[b] Diameter of induration 48 hr after intradermal injection of 50 µg of antigen in 0.1 ml of saline given on day 18.

[c] Log_2 of µg of antibody precipitated by antigen in 1 ml of serum. Sera were collected separately 3 weeks after treatment. They are tested separately, in experimental groups; the increase in titer is highly significant ($p < 0.01$) as compared with the control by Student's t test.

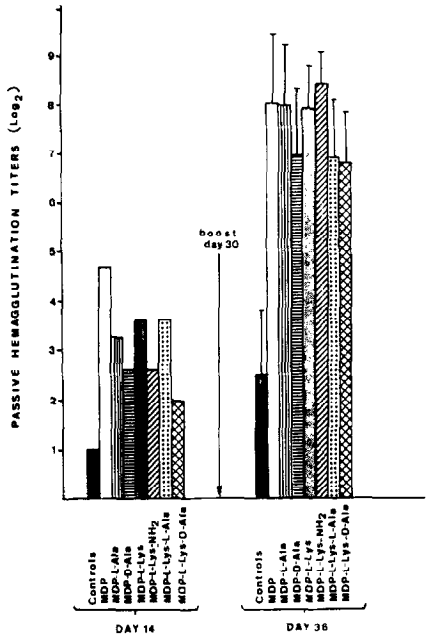

Figure 1. Adjuvant activity of MDP and analogs in mice. Animals (8 per group) received antigen in saline with or without adjuvant. Thirty days later they are boosted by antigen alone. Primary and secondary humoral antibody responses are estimated by passive hemagglutination. In all experimental groups the increase in titer is highly significant ($p < 0.01$) as compared with the control by Student's t test.

are expressed in a $\log_2$ scale and it can be seen that the titers in the experimental groups are more than 10 times higher than in the control group.

Adjuvant activity when injected into mice in saline. The data obtained in mice without the use of a water-in-oil emulsion confirmed the above results: no significant changes in activity were observed when either L-Ala, D-Ala, or peptides containing them, were linked to MDP. The results are summarized in Figure 1.

Enhancement of thymidine uptake in mouse spleen cells *in vitro* by MDP and analogs. It has been previously reported that the ability of MDP to stimulate thymidine incorporation was particularly prominent with spleen cells from certain high-responder mouse strain such as DBA/2 (13). The best results are obtained

when cells are incubated for 4-5 days in a 2-ME supplemented serum-free medium; MDP and its six analogs increased ^{3}H-thymidine uptake. These findings are in agreement with previous observations that adjuvant activity of monomeric MDP analogs in mice correlated well with their capacity to increase DNA synthesis in spleen cell cultures. Thus far, only one exception has been observed when a non-active MDP derivative has been cross-linked by glutaraldehyde (17).

Protective activity of MDP and analogs in mice infected with *K.pneumoniae*. The same glycopeptides were also tested for their capacity to stimulate nonspecific resistance to infections in an experimental model wherein MDP was shown effective (8). A hundred µg of each compound were administered to mice 24 hr before an intramuscular injection of 1.5×10^{4} *K.pneumoniae*. Survival was recorded for 10 days since usually no deaths occur after that time. Cumulative results of three identical experiments are reported in Fig. 2. Compounds containing a D-alanyl residue (MDP-D-Ala and MDP-L-Lys-D-Ala) are unable to protect mice, whereas the 4 others (MDP-L-Ala, MDP-L-Lys, MDP-L-Lys-NH_2 and MDP-L-Lys-L-Ala) are as effective as MDP ($P < 0.01$). Thus, it is shown that anti-infectious activity, in contrast with adjuvanticity, depends strongly on the stereochemistry of the added residue.

DISCUSSION

The results presented provide new possibilities of dissociating adjuvant activity from anti-infectious activity of synthetic glycopeptides by modifying their chemical structure. The importance of optical configuration of the first amino-acid residue has been previously reported; replacement of L-Ala by D-Ala results in a compound devoid of immune stimulatory properties (8), yet capable of decreasing certain immunological responses (6, 7).

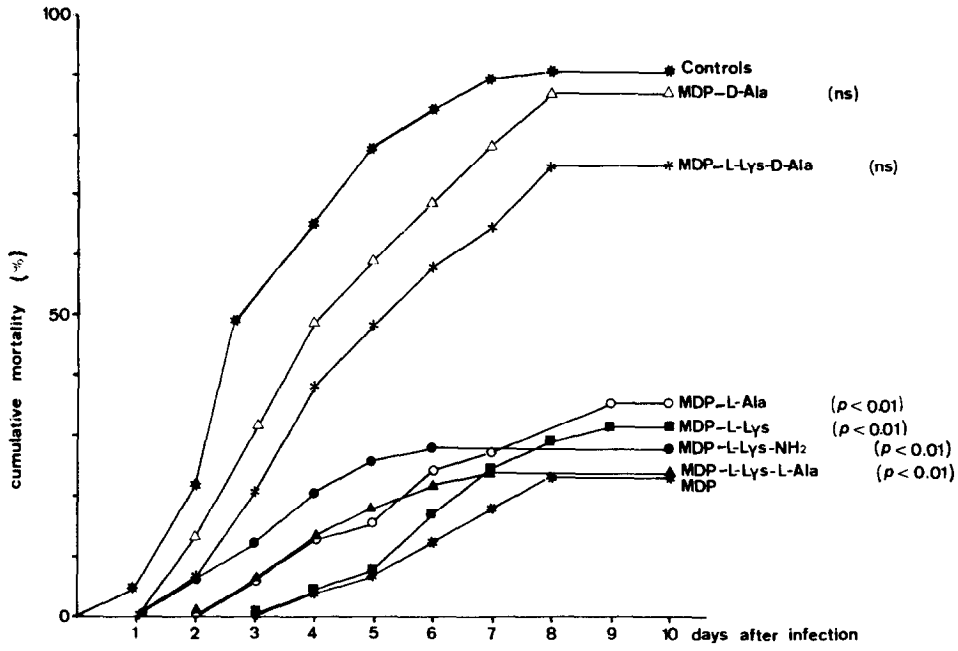

Figure 2. Effect of treatment with MDP and analogs in mice infected with 1.5×10^4 *Klebsiella pneumoniae*.

Synthetic MurNAc-L-Ala-D-Glu(L-Lys-D-Ala)-NH_2 which is the primary structure of many bacterial peptidoglycans, is adjuvant-active (3, 10, 12), and substitution of the carboxyl of D-isoGln by a L-Lys residue (4) can even increase its activity as compared to MDP (18). It is shown here that MDP-L-Lys-NH_2 (5) is also fully active. The data confirm that a good correlation exists between the capacity of monomeric glycopeptides to enhance lymphocyte thymidine incorporation and adjuvant activity. Furthermore, they show that adjuvant-active glycopeptides containing a D-alanyl residue at the C-terminal end of the peptide chain (3 and 7) do not enhance nonspecific resistance of mice to infectious challenge, in contrast to those containing L-alanine (2 and 6). It is clear that the lack of anti-infectious activity is due to the change of the optical configuration of the alanyl residue. It is not known if the active form is MDP and the presence of D-Ala prevents the

necessary degradation, or if the muramyl tripeptide or tetrapeptide are active only when the terminal amino-acid is L-Ala. These possibilities could be checked by the administration of radiolabelled compounds.

Adjuvant-active compounds devoid of anti-infectious activity can also be obtained by other chemical substitutions such as replacement of L-Ala with L-Ser in MDP, amidation or methyl-amidation of the carboxyl groups of the D-glutaminyl residue (19). The reverse situation has been observed also with certain lipophilic analogs not containing N-acetyl-muramic acid which retain their anti-infectious activity (20) although they are no longer adjuvant-active.

Acknowledgements : The skilful technical assistance of Ms Lydie Caille and Ms Béatrice Brunel is greatly acknowledged. We wish to thank Dr. Yi-chi M. Kong for the interesting discussions during the preparation of the manuscript. This work has been supported by INSERM grant C.R.L. 80-10.02 and by D.G.R.S.T. 78-7.28. 51.

REFERENCES

1. Ellouz, F., Adam, A., Ciorbaru, R., and Lederer, E. (1974) Biochem. Biophys. Res. Commun. 59, 1317-1325.
2. Schleifer, K.H. (1972) Bact. Rev. 36, 407-477.
3. Kotani, S., Watanabe, Y., Kinoshita, S., Shimono, T., Morisaki, I., Shiba, T., Kusumoto, S., Tarumi, Y., and Ikenaka, K. (1975) Biken J. 18, 105-111.
4. Merser, C., Sinaÿ, P., and Adam, A. (1975) Biochem. Biophys. Res. Commun. 66, 1316-1322.
5. Audibert, F., Chedid, L., Lefrancier, P., and Choay, J.(1976) Cell. Immunol. 21, 243-249.
6. Chedid, L., Audibert, F., Lefrancier, P., Choay, J., and Lederer, E. (1976) Proc. Natl. Acad. Sci. USA 73, 2472-2475.
7. Adam, A., Devys, M., Souvannavong, V., Lefrancier, P., Choay, J., and Lederer, E. (1976) Biochem. Biophys. Res. Commun. 72, 339-346.
8. Chedid, L., Parant, M., Parant, F., Lefrancier, P., Choay, J., and Lederer, E. (1977) Proc. Natl. Acad. Sci. USA 74, 2089-2093.
9. Damais, C., Parant, M., and Chedid, L. (1977) Cell. Immunol. 34, 49-56.
10. Audibert, F., Chedid, L., Lefrancier, P., Choay, J., and Lederer, E. (1977) Ann. Inst. Pasteur Paris 128C, 653-661.

11. Kotani, S., Watanabe, Y., Kinoshita, F., Morisaki,I., Kato,K., Shiba, T., Kusumoto, S., Tarumi, Y., and Ikenaka, K. (1977) Biken J. 20, 39-45.
12. Azuma, I., Sugimura, K., Taniyama, T., Yamawaki, M.,Yamamura, Y., Kusumoto, S., Okada, S., and Shiba, T. (1976) Infect. Immun. 14, 18-27.
13. Damais, C., Parant, M., Chedid, L., Lefrancier, P., and Choay, J. (1978) Cell. Immunol. 35, 173-179.
14. Snedecor, G.W. (1956) Statistical Methods, pp. 217-219, Iowa State Univ. Press, Ames, IA.
15. Lefrancier, P., Choay, J., Derrien, M., and Lederman, I. (1977) Int. J. Peptide Protein Res. 9, 249-257.
16. Lefrancier, P., Derrien, M., Lederman, I., Nief, F., Choay,J., and Lederer, E. (1978) Int. J. Peptide Protein Res. 11, 289-296.
17. Parant, M., Damais, C., Audibert, F., Parant, F., Chedid, L., Sache, E., Lefrancier, P., Choay, J., and Lederer, E. (1978) J. Infect. Dis. 138, 378-386.
18. Parant, M., Parant, F., and Chedid, L. (1978) Proc. Natl.Acad. Scie. USA 75, 3395-3399.
19. Chedid, L., Audibert, F., and Johnson, A.G. (1978) Progress in Allergy 25, 63-105.
20. Parant, M., Audibert, F., Chedid, L., Level, M.,Lefrancier,P., Choay, J., and Lederer, E. (1980) Infect. Immun. 27, 826-831.