CORRELATION OF STRUCTURE AND ADJUVANT ACTIVITY OF N-ACETYL MURAMYL-L-ALANYL-D-ISOGLUTAMINE (MDP), ITS DERIVATIVES AND ANALOGUES.

ANTI-ADJUVANT and COMPETITION PROPERTIES OF STEREOISOMERS.

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SUMMARY

The synthetic N-acetyl muramyl-dipeptide (MDP) 1 has been shown to be fully adjuvant active in a water-in-oil emulsion ; we now report a study on the adjuvant activity of 10 derivatives and analogues of MDP under similar conditions.

$NaBH_4$ reduction of MDP 1 leads to the inactive muramicitol dipeptide 2 ; β-elimination gives the lactyl-dipeptide 3, which seems to inhibit adjuvant activity. Shortening of the lactyl side chain of MDP gives nor-MDP, 4, which is less active. Amongst the analogues in which one amino acid of the dipeptide moiety is replaced by another one, the L-Ser analogue 5 is fully active, whereas replacement of L-Ala by Gly or of D-iso-Gln by D-Glu-OH, or D-Glu $(OMe)_2$, or D-Glu (OMe)-NH_2 gives less active compounds (6, 8, 9, 10).

The diastereoisomer 7 where L-Ala is replaced by D-Ala, shows an anti-adjuvant activity.

INTRODUCTION

Freund's complete adjuvant contains whole mycobacterial cells as essential component. Our efforts to elucidate the chemical structure responsible for their biological properties have led to the identification of N-acyl-muramyl-L-alanyl-D-isoglutamine as the minimal active structure (1, 3 - 5), the acyl being a glycolyl group in Mycobacteria, Nocardiae and in Micromonospo-

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Abbreviations: MDP: N-acetyl muramyl-dipeptide
FIA : Freund's incomplete adjuvant

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ra (see 6) whereas all other bacterial cell walls contain the N-acetyl derivative ; this latter muramyl dipeptide (MDP) (1) has been synthesized (2, 7, 15) and found to be fully adjuvant active (1-3). Kotani et al. (8) have also evaluated the adjuvant activity of various cell wall peptidoglycans and confirmed our results on the minimal structure required for adjuvant activity (9).

In our experiments MDP is added to an emulsion of Freund's incomplete adjuvant with a saline solution of ovalbumin ; it increases the level of antibodies against ovalbumin and induces delayed hypersensitivity to the antigen. Audibert et al. (10) have recently found that MDP can even show humoral adjuvant properties in saline, when injected in mice. It was also established that this activity could be elicited by the oral route and that an analogue had an immunosuppressive activity (13). We report here the results of our investigation on the effects of various structural modifications of MDP on biological activity.

MATERIAL AND METHODS

MurNac-L-Ala-D-isoGln (1) was reduced by treatment with a 50-fold excess of $NaBH_4$ in water at 4° for 24 hours. Reaction was stopped by acidification and the muramicitol dipeptide 2, purified by chromatography on Sephadex G 25, was characterized by its composition : MurOH:Ala:Glu (0.90 : 0.95 : 1).

The lactyl-dipeptide 3 was obtained by β-elimination from MDP by treatment with Na phosphate buffer 0.05 M at pH 12.5 for 2 hours at 37° (11), purified by preparative paper electrophoresis at pH 3.6 (same migration as glutamic acid) and characterized by its composition Ala:Glu (1.05 : 1).

The synthesis of the analogues 4 to 11 will be described separately by P. Lefrancier and J. Choay (in preparation) ; all compounds tested gave correct elementary analyses and were homogeneous on thin layer chromatography.

Biological tests were performed as described previously (4), using Hartley guinea pigs and ovalbumin as antigen.

RESULTS

The structures of the 10 derivatives and analogues of MDP-1 which we have prepared and tested are shown in Fig. 1.

An intact N-acetyl-muramyl structure seems to be necessary for full adjuvant activity : $NaBH_4$ reduction gives the inactive muramicitol dipeptide 2, whereas β-elimination leads to the lactyl-dipeptide 3, which decreased the response, as compared to the FIA controls (table 1).

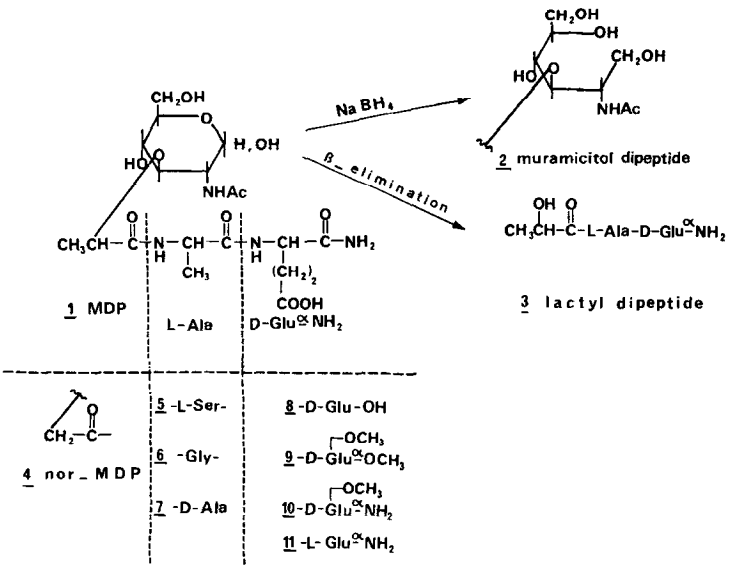

Figure 1 : Structure of N-acetyl Muramyl-L-Alanyl-D-Isoglutamine (MDP), its derivatives and analogues.

A synthetic "nor-MDP" 4, in which the lactyl side chain of MDP is replaced by a glycolyl side chain seems to be less active. We thus chose to keep the MurNac moiety of MDP as an invariant part of the molecule.

The α-carboxamide group is a critical feature in the active structure ; N-acetyl muramyl-L-Ala-D-Glu-OH 8 increases only very weakly the level of antibodies and is without effect on delayed hypersensitivity (10). This is in agreement with the low activity of the dimethyl ester (9) as compared to the γ-methyl ester of the isoglutamine derivative (10) (table 2).

In the analogues further synthesized the α-carboxamide group of glutamic acid was maintained and the effect of replacement of one amino acid of the active molecule by another residue was investigated. Table 2 shows that replacement of L-Ala by L-Ser (leading to 5) does not decrease the adjuvant effect, but compound 6 with Gly instead of L-Ala is much less active.

Inversion of the configuration of L-Ala leads to compound 7 which has an anti-adjuvant activity.

As can be seen in table 3, Mur-Nac-D-Ala-D-isoGln (7) added to Freund's complete adjuvant or to Mur-Nac-L-Ala-D-isoGln (1) behaves indeed as an antagonist of humoral response induced by the active adjuvant.

TABLE 1 : Action of various derivatives or analogs on the immune response.

Fraction tested	Dose (µg/animal)	Humoral antibodies (µg/ml) mean (a)	Delayed hypersensitivity to ovalbumin (b) (50 µg)
Freund's incomplete adjuvant	0	1503 ± 468	5 E to 4 I
Freund's complete adjuvant	50	3705 ± 981	15 I - 2 N to 5 N
MurNac-L-Ala-D-isoGln MDP 1	10	4787 ± 1600	15 I - 2 N to 5 N
Muramicitol Nac-L-Ala-D-isoGln 2	25	1922 ± 1115	5 I to 10 I - 2 N
Lactyl-L-Ala-D-isoGln 3	25	797 ± 441	0 to 5 I
nor MurNac-L-Ala-D-isoGln 4	25	4336 ± 1849	10 to 12 I -5 N
Mur Nac-L-Ala-D-Glu-OH 8	10	1764 ± 980	0 to 10 E - 2 I

The compounds (10 to 50 µg) were dissolved with 2, 5 mg of ovalbumin in 100 µl of saline and emulsified with 100 µl of Freund's incomplete adjuvant ; the emulsion was intradermally injected in both hind foot pads of Hartley female guinea pig weighing 350 g. Humoral antibodies against ovalbumin were determined 3 weeks after immunization by measurement of the antibody-antigen complex in the equivalence zone with Folin's reagent, on the sera obtained by cardiac puncture and delayed hypersensitivity to ovalbumin was determined by the skin reaction to 50 µg of ovalbumin intradermally injected in the flank of animals 4 weeks after sensitization.

a) 6 animals ; ± standard deviation.

b) The diameter of erythema (E), induration (I) or induration and necrosis (I-N) is given in mm, reaction measured at 48 hours.

TABLE 2 : Action of various MurNac dipeptides on the immune response.

Fraction tested	Dose (μg/animal)	Humoral antibodies (μg/ml) mean	Delayed hypersensitivity to ovalbumin (50 μg)
Freund's incomplete adjuvant	0	2293 ± 708	3 I to 4 I - I N
Freund's complete adjuvant	50	5775 ± 1899	10 I - 2 N to 12 I - 4 N
MurNac-L-Ala-D-isoGln 1	10	6513 ± 2186	10 I - 3 N to 14 I - 5 N
MurNac-L-Ser-D-isoGln 5	25	7200 ± 2082	8 I to 12 I - 5 N
MurNac-Gly-D-isoGln 6	25	2867 ± 1768	8 I to 10 I - 1 N
MurNac-D-Ala-D-isoGln 7	50	1522 ± 923	5 E to 6 I
MurNac-L-Ala-D-Glu-α, γ $(OCH_3)_2$ 9	25	1926 ± 637	15 E to 15 I - 4 N
MurNac-L-Ala-D-Glu(α-NH_2, γ-OCH_3) 10	25	4620 ± 1914	15 I - 3 N to 18 I - 8 N
MurNac-L-Ala-L-isoGln 11	50	1645 ± 1139	5 E to 6 I

See legend of Table 1.

Compounds 7 and 11 seem to be slightly inhibitory, but this effect is not statistically significant.

TABLE 3 : Inhibition of adjuvant activity by MurNac-D-Ala-D-isoGln.

Fraction tested	Dose (μg/animal)	Humoral antibodies (μg/ml) mean	Delayed hypersensitivity to ovalbumin (50 μg)
Freund's incomplete adjuvant	0	1162 ± 434	0 to 6 E
Freund's complete adjuvant	50	6975 ± 1732	10 I - 2 N to 15 I - 4 N
Freund's complete adjuvant + MurNac-D-Ala-D-isoGln 7	50 + 50	4227 ± 1872	10 I - 2 N to 12 I - 4 N
MurNac-L-Ala-D-isoGln 1	10	7717 ± 1068	15 I - 5 N to 20 I - 7 N
MurNac-L-Ala-D-isoGln + MurNac-D-Ala-D-isoGln 1 + 7	10 + 10	4916 ± 1754	12 I - 3 N to 15 I - 5 N
MurNac-L-Ala-D-isoGln + MurNac-D-Ala-D-isoGln 1 + 7	10 + 25	3400 ± 1588	12 I to 12 I - 4 N

See legend of Table 1.

DISCUSSION

Having shown previously that the naturally occurring N-acyl muramyl-L-alanyl-D-isoglutamine (MDP, 1) is a potent adjuvant (1-3 ; see also 10,13) we have investigated the influence of various analogues of this compound on the immune response.

The inactivity of the muramicitol dipeptide 2, the slight anti-adjuvant activity of the lactyl dipeptide 3 and the weaker activity of the nor-dipeptide 4 suggest that an intact N-acyl muramyl structure is essential ; we thus studied various structural modifications of the dipeptide moiety. Only the L-seryl analogue 5 is as active as the natural compound (MDP, 1) (and indeed, L-Ser replaces L-Ala in some peptidoglycans, (12)) ; the Gly analogue 6 (although also present in some peptidoglycans) is only weakly active.

The amide function of the C-terminal D-Glu seems to be necessary for full adjuvant activity in incomplete Freund's adjuvant (i.e. in presence of paraffin oil), as the diacid 8 is only weakly active under these conditions. Kotani et al. (9) have found compound 8 active, but Audibert et al. (10) have obtained results similar to our present findings. It is all the more remarkable that compound 8 is strongly active when injected in saline into mice, as discovered by Audibert et al. (10). The stronger adjuvant activity of the α-amide methyl ester 10, in comparison to the dimethyl ester 9 confirms the importance of the amide function (in Freund's incomplete adjuvant).

In parallel experiments, Chedid et al. (13) have tested compounds 9 and 10 on guinea pigs in FIA and obtained results analogous to ours. They have also tested analogues 5, 6, 8, 9, 10, 11 injected in saline, on the humoral response of mice to high and to low dosages of BSA ; they have shown that MDP (1) and compound 8 are even adjuvant active when given by the oral route.

As concerns the anti-adjuvant activity of some compounds we can distinguish two cases : in the first experiment (Table 1) where the antibody level of the FIA control is rather high, the lactyl-dipeptide 3 decreases the level of the control. Table 3 shows that the MurNac-D-Ala-D-Glu-α-NH_2 analogue 7 when added to either complete Freund's adjuvant or mixed with the active muramyl dipeptide (MDP) 1 decreases the humoral antibody response although delayed hypersensitivity seems not to be affected.

Chedid et al. (13) have also found that compound 7 (and even the water

soluble adjuvant WSA (4), under certain conditions) inhibits the immune response, when given to mice in saline. This might indicate a competition of the -D-D-analogue for the "receptor site" of the -L-D-compound, which could be on the macrophage, as Juy et al. (14) have shown that MDP (1) activates macrophages in vitro.

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