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Immunology

Modulation of the immune response by a synthetic adjuvant and analogs

(analogs of mycobacterial adjuvant/orally active adjuvant/inhibitors of immune response/Gram-negative lipopolysaccharide-low responder mice)

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ABSTRACT **N-Acetylmuramyl-L-alanyl-D-isoglutamine increases the humoral immune response of mice when given in aqueous media instead of the usual water-in-oil emulsions. Moreover, this compound is adjuvant active even by the oral route. In view of studying the relation between chemical structure and biological activity, several synthetic analogs were tested. The immune response could be modulated according to chemical modifications, and the synthetic analog with D- in place of L-alanine was shown to inhibit the immune response.**

It has been previously reported that water-soluble fractions can substitute for mycobacterial cells in Freund's complete adjuvant (FCA) (1–4). Subsequently, it was shown that synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine (AcMur-L-Ala-D-Glu-NH_2) has the minimal structure required to duplicate the activity of mycobacteria in Freund's complete adjuvant (5–7). More recently, we have reported that, in contrast to mycobacterial adjuvant preparations, the synthetic compound and a second synthetic analog, *N*-acetylmuramyl-L-alanyl-D-glutamic acid (AcMur-L-Ala-D-Glu) increase the humoral immune response when given in aqueous media instead of the usual water-in-oil emulsion (8). This finding led us to test several synthetic analogs administered with antigen to ascertain their activity on the immune response. They were administered with the antigen, either in saline to mice or in water-in-oil emulsion to guinea pigs.

In the work reported here, we also investigated the influence of various routes of administration of AcMur-L-Ala-D-Glu-NH_2 and its influence on mice that have a low response to lipopolysaccharide (LPS) from a Gram-negative bacterium (9). Moreover, the adjuvant activity of a series of synthetic compounds was evaluated after administration to guinea pigs in Freund's incomplete adjuvant (FIA) with ovalbumin.

MATERIALS AND METHODS

Animals. Except in one experiment in which the LPS low-responder C3H/He Orl. subline was used, mice were Swiss common stock. In all cases these mice were 2 months old and bred in the Centre National de la Recherche Scientifique Orléans Center. Male Hartley guinea pigs (Pasteur Institute) weighing 450 g were also used.

Preparations. The natural water-soluble adjuvant (WSA) was extracted from *Mycobacterium smegmatis* according to the procedure described by Adam *et al.* (10). LPS prepared from *Salmonella enteritidis* by the phenol-water procedure, FIA, and FCA were purchased from Difco Laboratories.

The synthetic analogs prepared by Lefrancier and Choay (unpublished results) were the following: *N*-acetylmuramyl-L-alanyl-D-isoglutamine (AcMur-L-Ala-D-Glu-NH_2); *N*-acetylmuramyl-L-alanyl-D-glutamic acid (AcMur-L-Ala-D-Glu); *N*-acetylmuramyl-L-seryl-D-isoglutamine (Ac-Mur-L-Ser-D-Glu-NH_2); *N*-acetylmuramyl-glycyl-D-isoglutamine (Ac-Mur-Gly-D-Glu-NH_2); *N*-acetylmuramyl-L-alanyl-L-isoglutamine (AcMur-L-Ala-L-Glu-NH_2); *N*-acetylmuramyl-L-alanyl-L-glutamic acid (AcMur-L-Ala-L-Glu); *N*-acetylmuramyl-D-alanyl-D-isoglutamine (AcMur-D-Ala-D-Glu-NH_2); methyl ester of *N*-acetylmuramyl-L-alanyl-D-isoglutamine [AcMur-L-Ala-D-Glu(OME)-NH_2]; dimethyl ester of *N*-acetylmuramyl-L-alanyl-D-glutamic acid (AcMur-L-Ala-D-Glu(OMe)-OMe]; 1-methyl amide of *N*-acetylmuramyl-L-alanyl-D-glutamic acid (AcMur-L-Ala-D-Glu-$NHCH_3$); L-alanyl-D-isoglutamine (L-Ala-D-Glu-NH_2).

The stability of the two methyl esters was tested by dissolving 2 mg in 0.5 ml of saline. Two hours later no hydrolysis was disclosed by silica gel thin-layer chromatography.

Antigens. These were purified preparations of egg albumin (crystallized five times) and bovine-serum albumin (BSA) Fraction V (Miles Laboratories).

Antibody Estimation. Anti-ovalbumin and anti-BSA were determined by passive hemagglutination using formalinized antigen-coated sheep erythrocytes (12). Anti-ovalbumin was also evaluated by quantitative precipitation, using Folin's method, and anti-BSA was measured by the antigen-binding Farr technique (13).

Delayed-Type Hypersensitivity. Guinea pigs were sensitized to ovalbumin according to the conditions described in *Results*. Eighteen days later, skin tests were performed intradermally, and 48 hr later diameters of induration were measured.

RESULTS

Influence of various routes of administration on adjuvant activity of AcMur-L-Ala-D-Glu-NH_2 administered to mice with BSA in saline

In these experiments, controls were immunized by subcutaneous or intravenous route with 0.5 mg of BSA and a boost of 0.1 mg of BSA 30 days later. Six experimental groups (8 mice each) received with the first injection of BSA either 0.1 mg of AcMur-L-Ala-D-Glu-NH_2 by subcutaneous or intravenous route, or higher dosages (0.1, 1, and 2 mg) of this adjuvant by oral route. A seventh group received 1 mg of AcMur-L-Ala-D-Glu, also by the oral route.

The data summarized in Table 1 make it evident that it is not necessary to administer the antigen and the adjuvant together

Abbreviations: FCA, Freund's complete adjuvant; LPS, lipopolysaccharide, from a Gram-negative bacterium; FIA, Freund's incomplete adjuvant; WSA, water-soluble adjuvant, extracted from *Mycobacterium smegmatis;* BSA, bovine-serum albumin. Abbreviations are used according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (11).

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Table 1. Adjuvanticity of AcMur-L-Ala-D-Glu-NH_2 and AcMur-L-Ala-D-Glu on the humoral response of mice to BSA administered in saline by the same or by different routes

Immunization	Adjuvant dose (mg)	Adjuvant route	Antigen route	Hemagglutination titer on Day 14	Day 28	Day 34	Day 36
Antigen (controls)	—	—	s.c.	<3	<3	<3	6
Antigen (controls)	—	—	i.v.	<3	<3	50	200
Antigen + AcMur-L-Ala-D-Glu-NH_2	0.1	s.c.	s.c.	25	50	400	1,620†
	0.1	i.v.	s.c.	12	12	200	1,010†
	0.1	s.c.	i.v.	50	50	100	790*
	2	p.o.	s.c.	6	12	200	1,350†
	1	p.o.	s.c.	6	6	200	850†
	0.1	p.o.	s.c.	<3	<3	25	50
Antigen + AcMur-L-Ala-D-Glu	1	p.o.	s.c.	3	6	100	140

Abbreviations used: s.c., subcutaneous; i.v., intravenous; p.o., by mouth (*per os*).

Eight mice per group received 0.5 mg of antigen and adjuvant when noted. At day 30, every mouse was boosted subcutaneously with 100 μg of antigen. At days 14, 28, and 34 titers were evaluated by passive hemagglutination on pooled sera for each group. At day 36, sera were collected separately. Significance was calculated by Student's *t* test, comparing experimental groups with their respective controls.

* $P < 0.05$.

† $P < 0.01$.

to observe an increase of the humoral immune response. Moreover, AcMur-L-Ala-D-Glu-NH_2 is active by various routes including administration by mouth. A smaller increase of the humoral response was observed when AcMur-L-Ala-D-Glu was administered orally.

Adjuvant activity of AcMur-L-Ala-D-Glu-NH_2 administered with BSA in saline to LPS low-responder mice

In these experiments, all mice were C3H/He Orl. and controls were immunized by subcutaneous route with 0.5 mg of BSA and a boost of 0.1 mg of BSA 30 days later. The experimental groups received either 0.1 mg of LPS or 0.1 mg of AcMur-L-Ala-D-Glu-NH_2 and were then bled at various intervals.

The data summarized in Table 2 show that, as expected, with the exception of one control and one LPS-treated mouse, all mice of these two groups were uniformly unresponsive to BSA. In contrast, an increased humoral response was observed in the animals treated with the synthetic adjuvant.

Adjuvant activity of various synthetic analogs administered to mice with a high dosage of antigen (0.5 mg of BSA) in saline

In these experiments, Swiss mice were immunized by the subcutaneous route as previously. The data summarized in Table 3 confirm our previous findings, i.e., that AcMur-L-Ala-D-Glu-NH_2 and AcMur-L-Ala-D-Glu had a strong adjuvant activity similar to that observed with their LPS controls. Moreover, their methyl esters and the 1-methyl amide of AcMur-L-Ala-D-Glu were equally active. The activity was also fully maintained when L-alanine was replaced by L-serine, whereas substitution by glycine decreased the adjuvanticity of this molecule [these two amino acids have been detected in certain corynebacterial peptidoglycans (14)]. In contrast, WSA and the other compounds tested in saline had very little or no demonstrable activity in this system.

Adjuvant activity of synthetic analogs administered to mice with a low dosage of antigen (0.05 mg of BSA) in saline

It was previously observed that if smaller dosages of BSA are administered and followed by a boost of 0.1 mg 30 days later, a weak but significant secondary response can be observed in the absence of adjuvant. In these same experiments it was also observed that in such cases WSA seemed to inhibit this response, whereas AcMur-L-Ala-D-Glu-NH_2 gave markedly increased levels of humoral antibody.

To confirm this effect and to investigate the possibility that some of the inactive analogs either had a marginal adjuvant effect or were capable of decreasing the immune response, the following experiments were performed. In this assay, all mice were immunized subcutaneously with 0.05 mg of BSA and a boost of 0.1 mg of BSA 30 days later. The experimental groups received by the same route 0.1 mg of the various analogs and 0.1 or 0.3 mg of WSA with the first injection of BSA.

The data summarized in Table 4 show that LPS, AcMur-L-Ala-D-Glu-NH_2, AcMur-L-Ala-D-Glu, and AcMur-L-Ser-D-Glu-NH_2 increased the antibody levels. In contrast, as can be seen by comparison with the controls, WSA and AcMur-

Table 2. Adjuvanticity of AcMur-L-Ala-D-Glu-NH_2 on the humoral response to BSA of LPS low-responder mice (C3H/He Orl.)

Immunization*	Hemagglutination titer on Day 14	Day 28	Day 34	Day 36
Antigen (controls)	<3	<3	<3	<3, <3, 200, <3, 3, <3, 3
Antigen + AcMur-L-Ala-D-Glu-NH_2	<3	12	200	200, 400, 200, 200, 200, 100, <3
Antigen + LPS	<3	<3	<3	12, 12, 3, 400, 25, 12, 12

At days 14, 28, and 34 titers were evaluated on pooled sera for each group. At day 36, figures represent individual results.

* Seven mice per group received subcutaneously: at day 1, 0.5 mg of BSA with or without 100 μg of adjuvant; at day 30, a boost of 100 μg of antigen.

Table 3. Comparison of the activities of different analogs of AcMur-L-Ala-D-Glu-NH_2 on the humoral response of mice to high dosages of BSA injected in saline

Immunization*	Hemagglutination titer on Day 14	Day 28	Day 34	Day 36
Antigen (controls)	<3	<3	<3	<3
Antigen + LPS	<3	6	50	1,310†
Antigen + AcMur-L-Ala-D-Glu-NH_2	6	6	200	1,620†
Antigen + AcMur-L-Ala-D-Glu	3	12	400	1,315†
Antigen + AcMur-L-Ser-D-Glu-NH_2	12	25	1,600	1,600†
Antigen + AcMur-Gly-D-Glu-NH_2	6	3	200	200
Antigen + AcMur-L-Ala-D-Glu(OMe)-NH_2	12	12	100	800†
Antigen + AcMur-L-Ala-D-Glu(OMe)-OMe	50	50	400	1,800†
Antigen + AcMur-L-Ala-D-Glu-$NHCH_3$	6	12	1,600	1,600†
Antigen + L-Ala-D-Glu-NH_2	<3	<3	<3	<3
Antigen + AcMur-D-Ala-D-Glu-NH_2	<3	<3	3	3
Antigen + AcMur-L-Ala-L-Glu	3	3	100	50
Antigen + AcMur-L-Ala-L-Glu-NH_2	3	6	50	25
Antigen + WSA	<3	<3	<3	6

At days 14, 28, and 34 titers were evaluated by passive hemagglutination on pooled sera for each group. At day 36, sera were collected separately; the median titer is shown. Significance was calculated by Student's *t* test.

* Eight mice per group received subcutaneously: at day 1, 0.5 mg of BSA with or without 0.1 mg of adjuvant; at day 30, a boost of 0.1 mg of antigen.

† $P < 0.01$.

D-Ala-D-Glu-NH_2 depressed the humoral response. All other compounds tested had little or no activity.

Adjuvant activity of synthetic analogs administered to guinea pigs with ovalbumin

Guinea pigs (groups of six) were given 0.1 ml of FIA emulsion containing 500 μg of ovalbumin in both posterior footpads. Control groups received either antigen in FIA only or the antigen in FCA, whereas the test groups received the antigen with either analog in FIA. The animals were skin-tested on day 18 and bled on day 21.

The results reported in Table 5 show that in contrast to AcMur-L-Ala-D-Glu-NH_2, compounds containing L-glutamic acid or L-isoglutamine are not adjuvant active: skin reactions were negative and the humoral response was not increased. These results are in agreement with previous reports of Kotani *et al.* (7). Methyl esters of AcMur-L-Ala-D-Glu-NH_2 and AcMur-L-Ala-D-Glu and the 1-methyl amide of the latter were also tested. As can be seen, the 1-methyl amide and the monomethyl ester elicited a very strong response, in contrast with the analog that contained two *O*-methyl groups.

DISCUSSION

It was previously reported that two synthetic adjuvants, AcMur-L-Ala-D-Glu-NH_2 and AcMur-L-Ala-D-Glu, administered subcutaneously in saline with antigen were capable of

Table 4. Comparison of activities of different analogs of AcMur-L-Ala-D-Glu-NH_2 on the humoral response of mice to low dosages of BSA

Immunization*	Titer† on Day 14 PA	Day 14 ABC	Day 28 PA	Day 28 ABC	Day 34 PA	Day 34 ABC	Day 36 PA	Day 36 ABC	Day 42 PA
Antigen (controls)	<3	<20	<3	<20	6	<20	50	25	25
Antigen + LPS	<3	<20	25	<20	400	300	3,200	500	800‡‡
Antigen + WSA (100 μg)	<3	<20	<3	<20	6	<20	3	<20	3§
Antigen + WSA (300 μg)	<3	<20	<3	<20	<3	<20	<3	<20	<3§§
Antigen + AcMur-L-Ala-D-Glu-NH_2	6	<20	25	20	400	210	1,600	500	550‡‡
Antigen + AcMur-L-Ala-D-Glu	3	<20	12	<20	200	80	800	400	605‡‡
Antigen + AcMur-L-Ser-D-Glu-NH_2	<3	<20	3	<20	50	<20	800	230	260‡
Antigen + AcMur-Gly-D-Glu-NH_2	<3	<20	<3	<20	12	<20	200	125	150
Antigen + L-Ala-D-Glu	<3	<20	3	<20	25	<20	200	115	120
Antigen + AcMur-L-Ala-L-Glu	<3	<20	6	<20	25	<20	200	100	170
Antigen + AcMur-L-Ala-L-Glu-NH_2	<3	<20	<3	<20	6	<20	50	30	60
Antigen + AcMur-D-Ala-D-Glu-NH_2	<3	<20	<3	<20	3	<20	<3	<20	<3§§

* Eight mice per group received subcutaneously: at day 1, 50 μg of BSA with or without 100 μg of adjuvant; at day 30, a boost of 100 μg of antigen.

† PA, passive hemagglutination; ABC, antigen binding capacity.

‡ $P < 0.05$ and ‡‡ $P < 0.01$: significantly elevated as compared to the controls.

§ $P < 0.02$ and §§ $P < 0.01$: significantly decreased as compared to the controls.

Table 5. Comparison of the activities of analogs of AcMur-L-Ala-D-Glu-NH_2 on the immune response of guinea pigs to ovalbumin in FIA

Immunization*	Skin reactions† to 5 μg ovalbumin, mm diameter	Antibodies against ovalbumin: Precipitation, μg/ml	Antibodies against ovalbumin: Agglutination titers
Antigen + FIA (controls)	0, 0, 0, 0, 0, 0	≤500	900
Antigen + FCA	12, 13, 12, 14, 11, 9	2,100	3,600
Antigen + FIA + AcMur-L-Ala-D-Glu-NH_2	12, 12, 10, 15, 12, 10	2,000	3,500
Antigen + FIA + AcMur-L-Ala-D-Glu(OMe)-NH_2	14, 8, 13, 12, 14, 11	2,100	3,000
Antigen + FIA + AcMur-L-Ala-D-Glu(OMe)-OMe	5, 7, 0, 8, 10, 7	950	2,600
Antigen + FIA + AcMur-L-Ala-D-Glu-$NHCH_3$	8, 9, 14, 15, 15	5,500	6,100
Antigen + FIA + AcMur-L-Ala-L-Glu-NH_2	0, 0, 0, 0, 0	650	1,040
Antigen + FIA + AcMur-L-Ala-L-Glu	0, 0, 0, 0, 0	700	1,550

* Each animal received 1 mg of ovalbumin. Adjuvant was administered at the dose of 100 μg.
† Reactions 48 hr after challenge on day 18.

increasing the humoral immune response in mice (8). In the present study the following findings were made:

(*a*) AcMur-L-Ala-D-Glu-NH_2 does not have to be administered with the antigen by the same route to exert its adjuvant effect. Moreover, surprisingly, this compound was active when administered orally. It can be tentatively assumed that activity by this route is related to the presence of D-isoglutamine which could render this molecule resistant to degradation by the mammalian host.

(*b*) The adjuvanticity of AcMur-L-Ala-D-Glu-NH_2 was maintained in the C3H/He Orl. subline, which does not respond to LPS. This observation is consistent with the previously reported effect of FCA in these same LPS-low-responder mice (15).

(*c*) Several other synthetic analogs were also tested in comparison. They were administered with the antigen either in saline to mice or in FIA to guinea pigs.

Substitution of D-isoglutamine by D-glutamic acid (i.e., elimination of the 1 amide group), as previously observed, did not affect the activity in saline. After replacement of L-alanine by L-serine or glycine adjuvanticity was maintained, although the substitution by glycine gave a much weaker effect. The 1 methyl amide and methyl ester derivatives of AcMur-L-Ala-D-Glu and the methyl ester of AcMur-L-Ala-D-Glu-NH_2, which were stable in aqueous solution *in vitro* (see *Materials and Methods*), were shown to be active when administered in saline to mice. However, in guinea pigs the analog that contains the two *O*-methyl groups had a very weak effect on both immune responses, whereas the two other compounds were markedly effective. These results show that esterification does not affect the expression of adjuvanticity in saline. The derivative in which both 1 and 5 carboxyls of glutamic acid are esterified has lost most of its activity when administered in a water-in-oil emulsion.

(*d*) When mice were immunized with lower dosages of antigen, an antibody response could be observed in the control groups. Under the same conditions, all analogs tested that were shown to be active in the previous high dosage BSA system retained their capacity to increase markedly the antibody titers as compared to those of the controls. In contrast, native WSA and one of the synthetic analogs, AcMur-D-Ala-D-Glu-NH_2, were shown to inhibit the immune response.

The data reported here demonstrate that synthetic AcMur-L-Ala-D-Glu-NH_2 retains its adjuvant activity even if administered in saline by the oral route. Moreover, the immune response can be inhibited by analogs of this molecule.

The peptidoglycan structure that can duplicate the activity of mycobacteria in FCA is very widely distributed in bacterial species, since it has been demonstrated not only in acid-fast but also in Gram-negative and Gram-positive organisms (14, 16, 17).

Since the minimal structural requirement is a muramyl dipeptide, it is possible to speculate whether there exists a similarity between these molecules and mammalian mediators capable of influencing the immune response.

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