CORRELATION OF STEREOCHEMICALLY SPECIFIC STRUCTURE IN MURAMYL DIPEPTIDE BETWEEN MACROPHAGE ACTIVATION AND ADJUVANT ACTIVITY

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Summary: N-acetylmuramyl-L-alanyl-D-isoglutamine, a synthetic substance of a minimal structure required for the adjuvant activity of bacterial cell walls was found to activate macrophages in mice, whereas its diastereomer, N-acetylmuramyl-L-alanyl-L-isoglutamine, which is inactive as adjuvant did not activate them.

Many microorganisms and their products exert immunological adjuvant activity, that is, a capacity to potentiate immune responses against various antigens including infectious agents and tumor cells. The most widely studied and used among them are tubercle bacilli (1,2). However, the mechanism of the activity remains largely unknown.

One of the promising approaches for elucidating the mechanism may be to purify active substances in the bacteria. The tubercle bacilli contain at least 3 adjuvant-active substances: wax D (peptidoglycolipid), cord factor (trehalose mycolate) and ribosomal RNA. Recently, the minimal structure required for the adjuvant activity of wax D or cell walls in guinea pigs was found to be N-acetylmuramyl-L-alanyl-D-isoglutamine (MurNAc-L-Ala-D-isoGln) (3,4). This has been confirmed also in mice and rats by several investigators (5-7).

In the present study the synthetic MurNAc-L-Ala-D-isoGln was found to be active as adjuvant and to stimulate the phago-

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cytic activity of macrophages in mice, wereas its diastereomer, N-acetylmuramyl-L-alanyl-L-isoglutamine (MurNAc-L-Ala-L-isoGln) was found to be inactive as adjuvant and not to stimulate the phagocytic activity.

MATERIALS AND METHODS

Animals: C3H female mice of 16 to 17 weeks of age and male mice 8 to 9 weeks of age, C57BL female mice 8 weeks of age and CF1 (outbred) female mice 22 to 23 weeks of age were obtained from Institute for Experimental Animals, Kyushu University.

Preparation of muramyl dipeptides: The muramyl dipeptides, MurNAc-L-Ala-D-isoGln and MurNAc-L-Ala-L-isoGln, were prepared as described previously (8).

Assay for adjuvant activity: Mice were injected into the left hind footpads with 0.025 ml of saline or phosphate-buffered saline pH 7.2 (0.057 M Na_2HPO_4, 0.018 M KH_2PO_4, 0.075 M NaCl) containing either 500 μg of bovine serum albumin (Sigma) or 100 μg of egg albumin (Sigma, Grade V) as antigen with or without 100 μg of the muramyl dipeptides as adjuvant. Twenty two days later booster injections were made into the same site (hind footpads) as the first injection, using 0.025 ml of saline or phosphate buffered saline pH 7.2 containing 100 μg of bovine serum albumin or 100 μg of egg albumin. Four and 8 days after the booster injections 0.03 ml of blood samples were obtained and antibody titers wre measured by passive hemagglutination according to the carbodiimide method (9).

Assay for phagocytosis: Two tenth ml of phosphate-buffered saline pH 7.2 containing 200 μg of the muramyl dipeptides with or without 100 μg of egg albumin were filtered through millipore filter membranes (pore size, 0.22 μ), divided into two equal portions and injected intravenously and almost at the same time intraperitoneally. Twenty four hours later, the phagocytic activity of macrophages was assayed by measuring the rate of clearance of colloidal carbon (C11/1431a, Günther Wagner, Hanover, Germany) by the reticuloendothelial system according to the technique of Biozzi et al (10). Phagocytic indices were determined by injecting intravenously carbon suspensions (16 mg/ml) at the rate of 1 ml per 100 g body weight, removing 0.03 ml of serial blood samples from the retro-orbital venous plexus 2, 4, 6, 9, and 12 minutes later, hemolyzed in 3.5 ml of 0.1 % Na_2CO_3 and determining carbon concentrations by Hitachi spectrophotometer with a red filter. The phagocytic index K was calculated from the slope of the line obtained by plotting the density readings against time on a semilogarithmic scale. The corrected phagocytic index α was calculated as

$$\alpha = \sqrt[3]{K} \times \frac{Wb}{Wsl}$$

(Wb=body weight, Wsl=combined weights of spleen and liver)
The means and standard deviations of the mean were determined for K and α which were statistically analyzed using Student's t test.

Table 1. Effect of muramyl dipeptides on antibody production*

Materials injected**	No. of mice	Hemagglutination titer*** at day 26	day 30 ($\log_2$)
Exp. 1			
BSA (saline)	6	<3	<3
BSA + MurNAc-L-Ala-D-isoGln(saline)	6	3.5 ± 0.76	5.0 ± 1.7
BSA (PBS)	6	<3	<3
BSA + MurNAc-L-Ala-D-isoGln(PBS)	6	4.8 ± 1.8	9.0 ± 1.0
Exp. 2			
EA (PBS)	6	<3	<3
EA + MurNAc-L-Ala-L-isoGln(PBS)	6	<3	<3
EA + MurNAc-L-Ala-D-isoGln(PBS)	6	4.7 ± 1.2	6.7 ± 1.2

* CF1 mice were used.
** BSA: bovine serum albumin 500 μg; 100 μg muramyl dipeptides were used.
EA : egg albumin 100 μg
PBS: phosphate-buffered saline pH 7.2
Antigen and adjuvant were injected either in saline or PBS.
*** Sera from 2 mice were pooled. Sheep red blood cells were used for the measurement of antibody in Exp. 1 and rabbit red blood cells in Exp. 2. Booster injections were made at day 22 into the same site (hind footpads) as the first injections.
Numbers represent the mean titer ± standard deviation.

RESULTS

Adjuvant activity of muramyl dipeptide

As shown in Table 1 the addition of MurNAc-L-Ala-D-isoGln to the protein antigens caused a significant enhancement of antibody production in CF1 mice. In contrast, no enhancement was observed with MurNAc-L-Ala-L-isoGln. Similar results were obtained with C3H and C57BL mice (Data not shown).

Macrophage activating activity of muramyl dipeptides

As shown in Table 2, in all the strains of mice tested the injection of MurNAc-L-Ala-D-isoGln caused a significant increase in the phagocytic activity of macrophages in the reticuloendo-

Table 2. Effect of muramyl dipeptide on carbon clearance by reticuloendothelial system of mice

Strain of mice	Exp. No.	Material* tested	No. of mice	Phagocytic index (mean±S.D. K	α
C3H female 16-17 wks	1	-	8	0.038 ± 0.008	6.1 ± 0.47
		L-D	10	0.066 ± 0.019	6.7 ± 0.67
				p < 0.005**	p < 0.05**
	2	-	8	0.045 ± 0.016	6.0 ± 0.66
		L-L	7	0.053 ± 0.016	6.4 ± 0.59
		L-D	7	0.176 ± 0.071	10.0 ± 1.26
				p < 0.001**	p < 0.001**
				p < 0.005***	p < 0.001***
	3	-	8	0.052 ± 0.007	6.5 ± 0.29
		L-L	8	0.046 ± 0.012	6.1 ± 0.48
		L-D	7	0.122 ± 0.031	8.5 ± 0.59
				p < 0.001**	p < 0.001**
				p < 0.001***	p < 0.001***
C3H male 8-9 wks	1	-	7	0.050 ± 0.014	6.1 ± 0.51
		L-L	6	0.052 ± 0.009	6.0 ± 0.22
		L-D	7	0.070 ± 0.008	6.6 ± 0.85
				p < 0.02**	
				p < 0.01***	
CF1 female 22-23 wks	1	-	6	0.045 ± 0.012	7.4 ± 0.39
		L-L	6	0.037 ± 0.007	6.7 ± 0.50
		L-D	5	0.071 ± 0.016	8.8 ± 1.01
				p < 0.02**	p < 0.02**
				p < 0.005***	p < 0.005***
C57BL female 8 wks	1	-	6	0.044 ± 0.012	6.1 ± 0.42
		L-L	6	0.057 ± 0.021	7.1 ± 0.60
		L-D	5	0.089 ± 0.023	8.0 ± 0.44
				p < 0.005**	p < 0.001**

* Materials were injected 24 hours before the clearance test i.v.&i.p
L-L: N-acetylmuramyl-L-alanyl-L-isoglutamine, 200 μg
L-D: N-acetylmuramyl-L-alanyl-D-isoglutamine, 200 μg

**Statistical significance of observed difference between control group and the group injected with L-D.

***Statistical significance of observed difference between the groups injected with L-L and L-D.
All the differences between the control group and the group injected with L-L were statistically not significant.

therial system as compared wtih the injection of MurNAc-L-Ala-L-isoGln or that of phosphate-buffered saline. In contrast, the injection of MurNAc-L-Ala-L-isoGln caused no such increase as

Table 3. Effect of muramyl dipeptide and egg albumin on carbon clearance by reticuloendothelial system of mice

Exp. No.	Material* tested	No. of** mice	Phagocytic index(mean ± S.D.) K	Phagocytic index(mean ± S.D.) α
1	EA	8	0.038 ± 0.004	5.1 ± 0.27
	EA + L-D	6	0.057 ± 0.013 ($p < 0.005$)	5.6 ± 0.67
2	EA	9	0.039 ± 0.006	6.2 ± 0.37
	EA + L-D	9	0.063 ± 0.008 ($p < 0.001$)	7.0 ± 0.32 ($p < 0.001$)

* EA: egg albumin, 100 μg
L-D: N-acetylmuramyl-L-alanyl-D-isoglutamine, 200 μg

** C3H female mice were used.
Materials were injected i.v. and i.p. as described in the text.

compared with that of phosphate-buffered saline alone.

A significant increase was also observed when MurNAc-L-Ala-D-isoGln was injected together with the protein antigen, egg albumin, as shown in Table 3.

DISCUSSION

Many microorganisms, in particular, mycobacteria, B. pertussis and C. parvum, or bacterial products such as lipopolysaccaride of gram-negative bacteria, all of which are potent immunological adjuvants are known to stimulate the phagocytic activity of macrophages as measured by the rate of clearance of particulate matters in the reticuloendothelial system of various animals (11-14).

Since macrophages play a crucial role in immune reactions, it has been felt by many investigators that the activation of macrophages by those bacterial adjuvants may be one of the mechanisms of their activity. However, this is not generally

accepted. For example, Weigle and his associates presented the view that the enhancement of phagocytosis caused by lipopolysaccharide from gram-negative bacteria is unrelated with its adjuvant activity (14).

In the present study MurNAc-L-Ala-D-isoGln, which possesses a minimal structure required for the adjuvant activity of bacterial cell walls was used together with its adjuvant-inactive diastereomer, MurNAc-L-Ala-L-isoGln, as a control and clear cut results were obtained concerning the relationship between macrophage activation and adjuvant activity. MurNAc-L-Ala-D-isoGln caused a significant increase in the rate of carbon clearance as compared with its diastereomer. The diastereomer inactive as adjuvant caused no such increase as compared with the phosphate-buffered saline control. This correlation of stereochemically specific structure in the muramyl dipeptides between macrophage activation and adjuvant activity suggests strongly that the observed enhancement of the phagocytic activity of macrophages by MurNAc-L-Ala-D-isoGln is related to its immunologically adjuvant-active property.

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REFERENCES

1. Freund, J. (1956) Fortsch. Tuberk. Forsch. 7, 130-148.
2. Mathé, G. (1973) Immunopotentiation, Ciba Foundation Symposium 18, p.305-330, Associated Scientific Publishers, Amsterdam.
3. Ellouz, F., Adam, A., Ciorbaru, R. and Lederer, E. (1974) Bioch. Biophys. Res. Comm. 59, 1317-1325.
4. Kotani, S., Watanabe, Y., Kinoshita, F., Shimono, T., Morisaki, I., Shiba, T., Kusumoto,S., Tarumi, Y., and Ikenaka, K. (1975) Biken J. 18, 105-111.
5. Azuma, I., Sugiyama, K., Taniyama, T., Yamasaki, M., Yamamura, Y., Kusumoto, S., Okada, S. and Shiba, T. (1976) Infect. and Immun. 14, 18-27.
6. Chedid, L., Audibert, F., Lefrancier, P., Choay, J., and Lederer, E. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2472-2475.

7. Tanaka, A., Saito, R., Sugiyama, K., Morisaki, I., Kotani,S. Kusumoto, S. and Shiba, T. (1977) Infect. and Immun. in press.
8. Kusumoto, S., Tarumi, Y., Ikenaka, K. and Shiba, T. (1976) Bull. Chem. Soc. Japan, 49, 533-539.
9. Johnson, H.M., Brenner, K. and Hall, H.E. (1966) J. Immunol. 97, 791-796.
10. Biozzi, G., Benacerraf, B. and Halpern, B.N. (1955) Brit. J. Expt. Pathol. 36, 226-235.
11. Nelson, D.S. (1969) Macrophages and Immunity, p.115-131, North-Holland Publishing Co., Amsterdam.
12. Nicol, T., Quantock, D.C. and Vernon-Roberts, B. (1966) Nature, 209, 1142-1143.
13. Howard, J.G., Scott, M.T. and Christie, G.H. (1973) Immunopotentiation, Ciba Foundation Symposium 18, p.101-120, Associated Scientific Publishers, Amsterdam.
14. Golub, E.S. and Weigle, W.O. (1967) J. Immunol. 98, 1241-1247.