BIKEN JOURNAL Vol. 19, 9-13, 1976

CORRELATION BETWEEN THE IMMUNOADJUVANT ACTIVITIES AND PYROGENICITIES OF SYNTHETIC *N*-ACETYLMURAMYL-PEPTIDES OR -AMINO ACIDS

SHOZO KOTANI, YOSHIRO WATANABE and TSUTOMU SHIMONO

Department of Microbiology, Osaka University Dental School, Joan-cho, Kita-ku, Osaka

KAZUHIRO HARADA

Laboratory of Microbiology, Osaka Prefectural Institute of Public Health, Nakamichi, Higashinari-ku, Osaka

TETSUO SHIBA and SHOICHI KUSUMOTO

Faculty of Science, Osaka University, Toyonaka, Osaka

KANAE YOKOGAWA and MITSUHARU TANIGUCHI

Research and Development Division, Dainippon Pharmaceutical Co., Suita, Osaka

(Received August 25, 1975)

SUMMARY A total of 14 different *N*-acetylmuramyl-peptides or -amino acids with or without configurations inherent to bacterial cell wall peptidoglycans were synthesized and their pyrogenicities on intravenous injection into rabbits were tested. *N*-Acetylmuramyl-peptides, and especially *N*-acetylmuramyl-L-alanyl-D-isoglutamine and *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysine, which were previously shown to be adjuvant-active in both induction of delayed-type hypersensitivity and stimulation of increased serum antibody levels to ovalbumin in guinea pigs, exhibited distinct pyrogenicity at as low dose as 16 μg per rabbit. However, none of the adjuvant-inactive analogues or diastereomers of the above *N*-acetylmuramyl-dipeptide or related compounds caused any significant febrile response in rabbits, even at a dose of 250 μg per animal.

INTRODUCTION

Some constituents of gram-positive bacteria are known to cause fever when injected intravenously in rabbits (Atkins and Freedman, 1963). Among the pyrogens derived from gram-positive bacteria, those of group A *Streptococcus pyogenes* have been the most extensively studied. Roberson and Schwab (1961) and Rotta and Bednář (1969) showed that purified preparations of cell walls or their peptidoglycans isolated from *S. pyogenes* and other streptococci elicited a reproducible febrile response in rabbits. These authors claimed

that the pyrogenicity, like the other pathogenic properties of streptococcal cell walls, was demonstrated exclusively by suspensions of finely disrupted cell walls or peptidoglycan (mucopeptide) fragments of an appropriate particle size. However, previous work (Hamada et al., 1971) revealed that the higher molecular weight fraction (a complex of degraded peptidoglycans and a group-specific C-carbohydrate) isolated by gel filtration from *S. pyogenes* cell walls which had been digested with *Flavobacterium* L-11 enzyme, caused high fever and decrease in the number of circulating leukocytes when injected intravenously into rabbits. This seemed to be the first time that a soluble preparation of the pyrogenic principle has been obtained from the cell walls of gram-positive bacteria.

One of the most remarkable biological properties of bacterial cell walls is its adjuvant activity. To elucidate the minimum structural entity essential for this adjuvant activity, a variety of *N*-acetylmuramyl-peptides or -amino acids have recently been synthesized and their immunoadjuvant activities assayed. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine was identified as the unit chemical structure required for distinct adjuvant activity in induction of delayed-type hypersensitivity and in stimulation of increased serum antibody levels to ovalbumin when administered to guinea pigs as a water-in-oil emulsion. The importance of the configuration of the glutamic acid residue or its amide in the above *N*-acetylmuramyl-dipeptide for manifestation of activity was also demonstrated (Kotani et al., 1975).

The present investigation was on whether synthetic *N*-acetylmuramyl-peptides or -amino acids exhibited pyrogenicity, and on whether there was any correlation between the chemical structures, immunoadjuvant activities and pyrogenicites of these compounds. The purpose of this study was to obtain information on the modes of action of the adjuvant-active *N*-acetylmuramyl-peptides so that the immunological responses of test animals to antigens could be controlled.

MATERIALS AND METHODS

1. *Preparation of N-acetylmuramyl-peptides or -amino acids*

These compounds were prepared by condensation of benzyl *N*-acetyl-4, 6-O-benzylidene-α-muramide with various peptide (or amino acid) benzyl esters by the dicyclohexylcarbodiimide—*N*-hydroxysuccinimide or ethylchlorocarbonate—*N*-methylmorpholine method and removal of the protecting groups by hydrogenolysis. The syntheses of these compounds were briefly described previously (Kotani et al., 1975) and will be reported in detail elsewhere (Kusumoto et al., to be published).

2. *Pyrogenicity test and leukocyte count*

The procedure used was essentially that described in U.S. Pharmacopeia XVIII. The test specimen was dissolved in pyrogen-free physiological saline solution at an appropriate concentration, and filtered through a Swinnex®-25, sterilized filter unit (0.22 μm pore size, Millipore Corp., Mass., USA). A 10 ml volume of the solution containing the required dose for the test was injected intravenously into Japanese domestic rabbits, weighing 1.5 kg to 2.7 kg. To test pyrogenicity, the rectal temperature was measured continuously with an automatic body temperature-recording device (either Model TE-3, Ellab Co., Copenhagen, Denmark or Model EP-670, Iio Electrical Equipment Co., Tokyo). When more than two of the three rabbits tested showed an individual rise in temperature of 0.6 C or more above their control temperature, the material under examination was judged to be definitely pyrogenic. When none of the three rabbits showed a rise in temperature of 0.6 C or more, the test material was regarded as non-pyrogenic. When one of the three rabbits showed a temperature rise of 0.6 C or more, or when the sum of the temperature rises exceeded 1.4 C, the test specimen was judged to be weakly or doubtfully pyrogenic.

In some experiments leukocyte counts were made on free flowing blood samples taken from the marginal ear vein, using an Automatic Blood Cell Counter (Model MCC T. M., Type CC1002B, Tōa Electric Co., Tokyo), following the procedures described in the operating manual of the counter.

RESULTS

The changes in rectal temperature and the

number of leukocytes in the blood of individual rabbit after intravenous injection of 250 μg of either *N*-acetylmuramyl-L-alanyl-D-isoglutamine or *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine with adjuvant activity are shown in Figs. 1 and 2, respectively. In both rabbits, there was a rapid and marked increase in temperature and the count of circulating leukocytes first decreased, then increased and finally returned to the initial level, as after intravenous injection of either the cell walls of *S. pyogenes* or their enzymatic digests (Hamada et al., 1971). The fever response seemed to be biphasic in the rabbit injected intravenously with *N*-acetylmuramyl-L-alanyl-D-isoglutamine. This was like the well-known biphasic, febrile response of rabbits after intravenous injection of endotoxic lipopolysaccharides from gram-negative bacteria (Atkins and Snell, 1965). On the other hand, after intravenous injection of *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine, rabbits showed a monophasic rise in temperature, at least in the present experimental conditions.

Table 1 summarizes the results of pyrogenicity tests on a variety of *N*-acetylmuramyl-peptides and -amino acids, with and without adjuvant activity (Kotani et al., 1975). It is evident that there is an almost perfect positive correlation between the pyrogenicities and the immunoadjuvancies of these *N*-acetylmuramyl derivatives. However, it should be pointed out that the pyrogenicity tests were performed on rabbits, while the immunoadjuvant assays were made with guinea pigs.

DISCUSSION

It has been reported that the pyrogenicity of the cell walls of gram-positive bacteria may be primarily attributable to the peptidoglycan subunits, and in particular to the characteristic

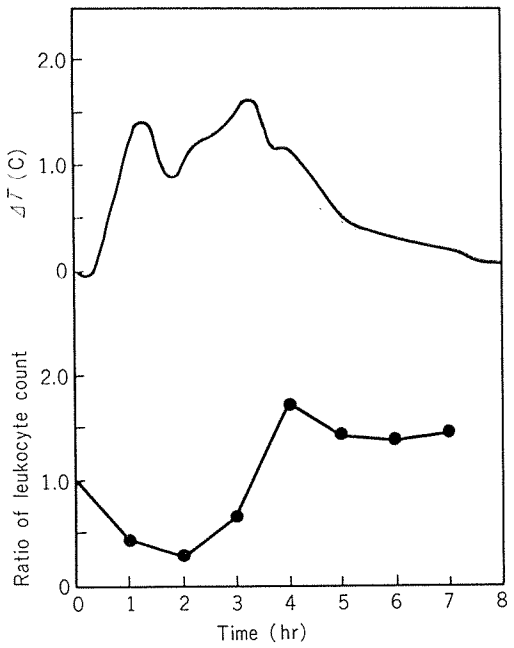

FIGURE 1. Changes in body temperature and leukocyte count following intravenous injection of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (250 μg/rabbit).

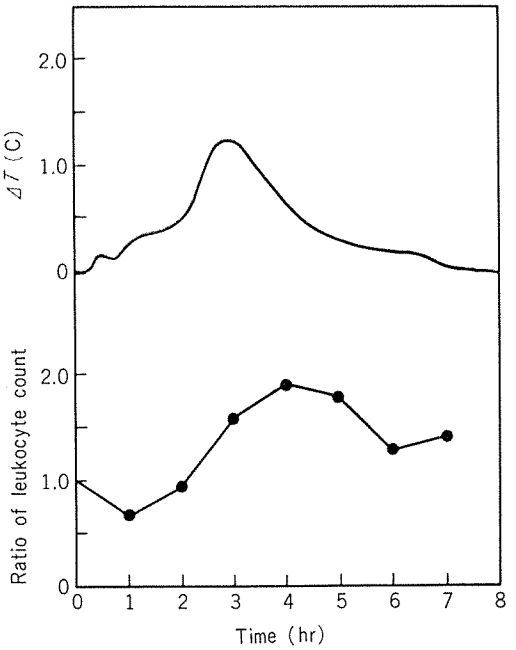

FIGURE 2. Changes in body temperature and leukocyte count following intravenous injection of *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (250 μg/rabbit).

TABLE 1. *Pyrogenicity of Synthetic N-Acetylmuramyl-Peptides and -Amino Acids — Correlation between Pyrogenicity and Immunoadjuvancy*

Test material	Adjuvancy[a]	Pyrogenicity (ΔT C)[b] Dose (μg)						
		250	125	62.5	31.3	15.6	7.8	3.9
MurNAc-L-Ala-D-Glu-NH$_2$ (L-Lys-D-Ala-OH)	3+	1.1	0.30	0.37	0.23	0.27		
MurNAc-L-Ala-D-Glu-NH$_2$ (L-Lys-OH)	3+	1.2	0.97	0.53	0.73	0.80	0.25	
MurNAc-L-Ala-D-Glu(OH)-NH$_2$	3+	1.5	1.3	0.90	0.87	1.0	0.37	0.22
MurNAc-L-Ala-L-Glu(OH)-NH$_2$	0	0.10	0.07	0.03				
MurNAc-L-Ala-D-Glu(NH$_2$)-OH	0	0.53	0.30	0.10				
MurNAc-L-Ala-L-Glu(NH$_2$)-OH	0	0.10	0.20	0.07				
MurNAc-L-Ala-D-Glu(OH)-OH	+	0.37	0.30	0.30	0.07	0		
MurNAc-L-Ala-L-Glu(OH)-OH	0	0.17	0.30	0.17				
MurNAc-L-Ala-D-Asp(OH)-NH$_2$	0	−0.07						
MurNAc-L-Ala-D-Ala-NH$_2$	0	0.03						
MurNAc-L-Ala-OH	0	0.33	0.23	0.10				
MurNAc-D-Ala-OH	0	0.20						
MurNAc-D-Glu(OH)-NH$_2$	0	0	0.23	0				
H-L-Ala-D-Glu-NH$_2$ (L-Lys-D-Ala-OH)	0	0.10	0.30	0.17				

[a] In both induction of delayed-type hypersensitivity and stimulation of increased serum antibody levels to ovalbumin when administered to guinea pigs as water-in-oil emulsions (Quoted from Kotani et al., 1975).

[b] ____: Definitely pyrogenic, ...: Weakly or doubtfully pyrogenic (See text for details. Three rabbits each were tested with each dose of test materials).

and common structure, *N*-acetylmuramyl-L-alanyl-D-isoglutamine. The possibility that the observed pyrogenicity of synthetic compounds was due to contaminating exogeneous endotoxic lipopolysaccharides seems to be inconsistent with the very close correlations observed between the pyrogenicities, immunoadjuvant activities and chemical structures of the test specimens.

The definite, but weaker fever response of rabbits after injection of *N*-acetylmuramyl-tetrapeptide than after injection of *N*-acetylmuramyl-L-alanyl-D-isoglutamine may be related partly to delay in the febrile response in the former case, and partly to the fact that at equal doses on a weight basis the molar concentration of *N*-acetylmuramyl-tetrapeptide was less than that of *N*-acetylmuramyl-dipeptide.

From the present result it is very tempting

to speculate that there may be some connection between the mechanisms controlling the immunological responses of mammals to an antigenic stimulus and the mechanisms regulating the body temperature or febrile response, and that some target(s) affected by the adjuvant-active *N*-acetylmuramyl-peptides may be involved in both these mechanisms.

ACKNOWLEDGMENTS

This research was supported in part by a Grant in Aid for Scientific Research from the Ministry of Education, Science and Culture (No. 911213) and by a Naito Research Grant for 1972 (No. 72–109).

REFERENCES

Atkins, E., and L. R. Freedman. 1963. Studies in staphylococcal fever. I. Responses to bacterial cells. Yale J. Biol. Med. 35: 451–472.

Atkins, E. and E. S. Snell. 1965. Fever. p. 495–534. *In* B. W. Zweifach and R. T. McCluskey [ed.] The inflammation process, Academic Press, New York.

Hamada, S., T. Narita, S. Kotani, and K. Kato. 1971. Studies on cell walls of group A *Streptococcus pyogenes*, type 12. II. Pyrogenic and related biological activities of the higher molecular weight fraction of an enzymatic digest of the cell walls. Biken J. 14: 217–231.

Kotani, S., Y. Watanabe, F. Kinoshita, T. Shimono, I. Morisaki, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka. 1975. Immunoadjuvant activities of synthetic *N*-acetylmuramyl-peptides or -amino acids. Biken J. 18: 105–111.

Roberson, B. S., and J. H. Schwab. 1961. Endotoxic properties associated with cell walls of group A streptococci. J. Infect. Dis. 108: 25–34.

Rotta, J., and B. Bednář. 1969. Biological properties of cell wall mucopeptides of hemolytic streptococci. J. Exp. Med. 130: 31–47.

Schwab, J. H., and S. H. Ohanian. 1968. Biological properties related to chemical structure of the streptococcal cell. p. 80–88. *In* R. Caravano [ed.] Current research on group A Streptococcus. Excerpta Medica Foundation, Amsterdam.