MICROBIAL PATHOGENECITY

Induction of necrosis factor- α and interleukin-6 in mice *in vivo* and in murine peritoneal macrophages and human whole blood cells *in vitro* by *Micrococcus luteus* teichuronic acids

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Earlier studies showed that *Micrococcus luteus* cells and cell walls induced anaphylactoid reactions leading to death, in some instances within 1 h, in C3H/HeN mice primed with muramyl dipeptide (MDP). They also induced serum cytokines in the surviving mice. The present study investigated the structural components responsible for these activities. Teichuronic acids, a component of *M. luteus* cell walls, induced tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in MDP-primed C3H/HeN mice. Peptidoglycans had little effect on the cytokine-inducing activities. Reducing teichuronic acids, i.e., teichuronic acids whose carboxyl groups had been reduced, lost their cytokine-inducing activities. Neither peptidoglycans nor teichuronic acids induced anaphylactoid reactions in the MDP-primed mice. Purified teichuronic acids also induced TNF- α and IL-6 production in C3H/HeN murine peritoneal macrophages and human whole-blood cells in the culture, but reduced teichuronic acids did not. The purified teichuronic acids induced no TNF- α and only low levels of IL-6 in MDP-primed C3H/HeJ mice, and neither cytokine in peritoneal macrophage cultures from C3H/HeJ mice with a single point of mutation in Toll-like receptor 4 (TLR4) gene. These findings suggest that induction of cytokines by teichuronic acids is mainly TLR4-dependent.

Introduction

Micrococcus luteus (*lysodeikticus*) was initially isolated from blood-agar cultures of the nasal secretions of a patient with acute coryza [1]. Human skin is now considered to be a primary habitat of this bacterium and it has also been detected in the mucous membranes, including the buccal cavity, as well as in water and soil [2]. Although *M. luteus* is usually disregarded as a contaminant in clinical specimens, it has been implicated in bacteraemia [3], septic shock [4], septic arthritis [5], endocarditis [6–8], meningitis [9], intracranial suppuration [10] and pneumonia [11].

A previous study [12] showed that *M. luteus* cells and cell walls caused anaphylactoid reactions leading to death, in some instances within 1 h, in C3H/HeN mice primed with *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP). It also demonstrated that the cells and cell walls induced tumour necrosis factor (TNF) and interleukin-6 (IL-6) in the serum of the surviving mice. *M. luteus* cell walls consist of two polymers, peptidoglycans [13, 14] and teichuronic acids [15–17], covalently bonded to each other. Peptidoglycans are generally known to be potent immunostimulants and to induce various cytokines *in vivo* and *in vitro* [18]. However, the immunoadjuvant activities of peptidoglycans of *M. luteus* in guinea-pigs were reported to be weak compared with those of other bacterial peptidoglycans [19, 20]. It was also reported that bacterial peptidoglycans showed low cytokine-inducing activities in murine macrophage culture [21]. The immunomodulating activities of teichuronic acids have not been studied.

The present study investigated which components of

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the cell walls could cause anaphylactoid reactions or induced cytokines such as TNF and IL-6 in MDP-primed C3H/HeN mice.

Materials and methods

Bacterial strains

M. luteus strains IFO 3333, MT and NCTC 2665 were mainly used. Strain IFO 3333 was supplied by the Institute for Fermentation, Osaka, Japan. Strain MT is an autolysis-defective, tetrad-forming mutant derived from strain IFO 3333, and MT/T is not a strain but cell packets formed from MT cells by treatment with proteases [22]. Strains NCTC 2665, also a type strain supplied by the National Collection of Type Cultures, Colindale Avenue, London, is autolysis-defective [23].

Peptidoglycans and glycans

Cell walls were prepared as described previously [23] from log-phase cells (LC) and stationary phase cells (SC), and they were designated as LCW and SCW, respectively. Peptidoglycans were prepared from cell walls as described previously [24]. LCW and SCW were subjected to mild acid treatment (0.1 N HCl, 100°C, 10 min) and centrifuged at 30 000 *g* for 30 min. The precipitate consisting of peptidoglycans was designated as PG (LCW) and PG (SCW), respectively. The mild acid treatment of the cell walls greatly decreased the teichuronic acid content of the cell walls [25–27]. The glycans were prepared from whole cells as described previously [28]. Stationary-phase cells of strain NCTC 2665 were refluxed with hydrazine hydrate 100% for 5 h or 20 h. The resulting samples were *N*-re-acetylated and designated as G 5 (NCTC 2665) and G 20 (NCTC 2665), respectively. G 5 (NCTC 2665) and G 20 (NCTC 2665) are polymers mainly composed of *N*-acetyl muramic acid and *N*-acetyl glucosamine residues, but the former residues are partly destroyed, to a greater extent in G 20 (NCTC 2665) than in G 5 (NCTC 2665). G 5 (NCTC 2665) has a few amino acids which are components of peptidoglycans and G 20 (NCTC 2665) has practically no amino acids.

Teichuronic acids

Teichuronic acids were prepared from cells as described previously [24]. LCW and SCW were digested with lysozyme (Lysozyme from chicken egg-white, 3 × crystallised, dialysed and lyophilised; Sigma) in 0.1 M sodium acetate buffer, pH 6.0, at 37°C for 20 h and dialysed against distilled water. The dialysate was designated as TUA (LCW) and TUA (SCW).

Purified teichuronic acids (p-TUA) and reduced teichuronic acids (p-TUA-[H])

p-TUA and p-TUA-[H] were prepared as described previously [15]. Briefly, cell walls prepared from SC

(NCTC 2665), i.e., SCW (NCTC 2665) were digested with egg-white lysozyme and further with L₁₁ enzyme. The digest was dialysed against distilled water and fractionated by an ECTEOLA-cellulose column. The fractionated sample was designated p-TUA. To convert the aminohexuronic acid residues of p-TUA to aminohexose residues, p-TUA were acetylated with acetic anhydride, reduced with diborane and treated with mild alkali (0.01 N NaOH, at room temperature, overnight) to remove *O*-acetyl groups, and the sample thus obtained was designated as p-TUA-[H]. The reduction of the carboxyl groups of the aminohexuronic acid residues to alcohol was confirmed by comparison of the infrared spectrum of p-TUA with that of p-TUA-[H]. The chemical structures of p-TUA and p-TUA-[H] are shown below [15, 16].

The lipopolysaccharide(LPS)-like content in PG (NCTC 2665, SCW), TUA (NCTC 2665, SCW), p-TUA and p-TUA-[H] was 0.151 ng/mg, 0.226 ng/mg, 0.091 ng/mg and 0.070 ng/mg, respectively, based on the results of the colorimetric *Limulus* test with *Escherichia coli* O111:B4 LPS as a control in an Endospecy Test (Seikagaku Kogyo, Tokyo, Japan) [29].

Chemical treatment of whole cells

Whole cells such as LC (IFO 3333) were subjected to mild acid treatment and mild Smith degradation [30], and the resulting samples were designated as, e.g., Mild Acid LC (IFO 3333) and Mild Smith LC (IFO 3333). These treatments greatly decreased the content of teichuronic acids in the cell walls and whole cells [25–27].

All the materials described above were lyophilised and used as test specimens.

Other reagents

MDP was supplied by Daiichi Pharmaceutical (Tokyo, Japan). Purified LPS prepared from *Salmonella choleraesuis* subsp. *choleraesuis* serotype abortus equi (*S. abortus-equi*) was supplied by C. Galanos (Max-Planck-Institut für Immunbiologie, Freiburg, Germany) [31]. Poly-L-lysine hydrobromide (MW 30 000–70 000) and poly-L-glutamic acid sodium salt (MW 15 000–50 000) were purchased from Sigma.

Induction of anaphylactoid reaction and serum cytokines in mice

The experimental method was described previously [12]. Briefly, groups of three-to-six C3H/HeN or C3H/HeJ mice (6–12-week-old males and females) were primed with an intravenous (i.v.) injection of

MDP (100 μg in 0.2 ml of pyrogen-free physiological saline). Then 4 h later, the mice received an i.v. injection of test specimen dissolved or homogeneously suspended in 0.2 ml of saline, and were observed for 1 h to determine the incidence of anaphylactoid reaction and death. At 90 min after the injection of the test specimen, surviving mice were bled from the subaxillary vein under ether anaesthesia to obtain test sera. All protocols used in this study were approved by the Animal Research Committee of Kagoshima University Dental School.

Induction of cytokines in murine peritoneal macrophage cultures

The experimental method was described previously [32]. Briefly, thioglycolate medium-induced peritoneal macrophages ($10^5/100\ \mu\text{l}$ /well of a flat-bottomed microculture plate) from C3H/HeN or C3H/HeJ mice were cultured in RPMI 1640 medium with or without test specimens at 37°C for 24 h. The cytokine activities of the culture supernate were measured as described below.

Induction of cytokines in human peripheral whole-blood cell cultures

The experimental method was described previously [32]. Briefly, heparinised peripheral blood was obtained from a healthy adult volunteer after informed consent. The blood (25 μl) was directly (without fractionation) incubated with test specimens in RPMI 1640 medium (100 μl) at 37°C for 24 h. Triplicate culture supernates were assayed for cytokines.

Measurement of cytokines

The TNF- α and IL-6 activities in the murine serum and murine macrophage and human whole-blood culture supernates were measured by enzyme-linked immunosorbent assay (ELISA) kits for murine and human TNF- α and IL-6 (Biosource International, Camarillo, CA, USA).

Results

Activities of peptidoglycans and glycans in MDP-primed C3H/HeN mice

Cell walls from strain NCTC 2665 as reference materials induced anaphylactoid reactions and early death within 1 h at incidences of 100% and 100% at a dose of 500 μg , and 33% and 0% at a dose of 100 μg , respectively. Other materials including peptidoglycans and glycans failed to induce anaphylactoid reactions in MDP-primed C3H/HeN mice. As shown in Fig. 1, peptidoglycans prepared from the stationary-phase cells of strains NCTC 2665, at a dose of 500 μg , induced low levels of TNF- α in mice, but other peptidoglycans and glycans did not. Peptidoglycans induced low levels

of IL-6 at a dose of 500 μg , and the glycan preparations showed practically no activity. It should be noted that MDP alone induced neither anaphylactoid reactions nor serum cytokines in C3H/HeN mice even at a dose of 5 mg.

Cytokine-inducing activities of teichuronic acids in MDP-primed C3H/HeN mice

The teichuronic acid preparation failed to induce an anaphylactoid reaction in the MDP-primed C3H/HeN mice (Fig. 1). Among the teichuronic acid preparations, those prepared from strains B 361 and NCTC 2665 induced both serum TNF- α and IL-6 in MDP-primed C3H/HeN mice. It should be noted that other inactive or low activity teichuronic acid preparations in Fig. 1 were derived from cell-wall preparations lacking cytokine-inducing activities [12]. The cytokine-inducing activities of the teichuronic acids derived from strain NCTC 2665 were maintained after purification (p-TUA). In contrast, reduced teichuronic acids (p-TUA-[H]) induced neither TNF- α nor IL-6. When p-TUA (100 μg) was injected into non-primed C3H/HeN mice, practically no TNF- α and only low levels of IL-6 were detected.

Effect of chemical treatments of whole cells on activities in MDP-primed C3H/HeN mice

Whole cells subjected to mild acid treatment and mild Smith degradation induced anaphylactoid reactions, but they induced no TNF- α and little IL-6 (Fig. 2).

Cytokine-inducing activities of mixtures of teichuronic acids and peptidoglycans or glycans in MDP-primed C3H/HeN mice

As shown in Fig. 1, the mixture of p-TUA and PG (NCTC 2665, SCW) or G5 (NCTC 2665) or G20 (NCTC 2665) showed cytokine-inducing activities that were significantly lower than those of p-TUA alone in the serum of MDP-primed C3H/HeN mice. The mixture of teichuronic acids and peptidoglycans or glycans did not cause anaphylactoid reactions and death in mice.

Effect of addition of poly-L-lysine and poly-L-glutamic acid and MDP on cytokine-inducing activities of teichuronic acids in MDP-primed C3H/HeN mice

The mixture of poly-L-lysine and p-TUA, which is slightly turbid, induced less TNF- α and IL-6 than p-TUA alone in mice. In contrast, the mixtures of poly-L-glutamic acid or MDP and p-TUA induced significantly less TNF- α than p-TUA alone and significantly more IL-6 than p-TUA alone in mice. (Fig. 3).

Fig. 1. Induction of serum cytokines by peptidoglycans, glycans, teichuronic acids and their mixtures in MDP-primed C3H/HeN mice. Groups of three-to-six C3H/HeN mice were primed with an i.v. injection of MDP (100 μ g). Then 4 h later, the mice received an intravenous injection of test specimen and they were observed for 1 h to record the incidence of anaphylactoid reaction and death. At 90 min after the injection of the test specimen, surviving mice were bled to obtain test sera. The serum TNF- α and IL-6 levels were measured by ELISA and their values are expressed as means and SEM (left and right columns, respectively). ‘Died’ means that all the mice in the group died within 1 h after the injection of the test material. ‘*Teichuronic acids + Peptidoglycans or Glycans*’ were examined by Student’s *t* test, indicating significant difference from the respective ‘p-TUA’ (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Fig. 2. Induction of anaphylactoid reactions in MDP-primed C3H/HeN mice by whole cells treated with reagents. Procedures were the same as those described in Fig. 1. The incidences of anaphylactoid reaction (□) and death (■) are shown. No TNF- α and little IL-6 activity was induced by these cells in the sera of the mice.

Fig. 3. Effects of addition of poly-L-lysine, poly-L-glutamic acid and MDP on cytokine-inducing activities of teichuronic acids in MDP-primed C3H/HeN mice. Procedures were the same as those described in Fig. 1. At a dose of 100 µg of poly-L-lysine alone, one of three MDP-primed C3H/HeN mice had an anaphylactoid reaction and died. The injection of 500 µg of poly-L-lysine alone or of the mixture of 500 µg of poly-L-lysine and 100 µg of p-TUA caused anaphylactoid reactions and death in all three mice. 'Mixed materials' were examined by Student's *t* test, indicating significant difference from the respective 'p-TUA' (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Cytokine-inducing activities of teichuronic acids in peritoneal macrophage cultures from C3H/HeN mice

p-TUA induced TNF- α and IL-6 in peritoneal macrophage cultures from C3H/HeN mice (Fig. 4). In contrast, p-TUA-[H] was scarcely active in this respect.

Cytokine-inducing activities of teichuronic acids in C3H/HeJ mice

p-TUA and p-TUA-[H] failed to induce anaphylactoid reactions in MDP-primed C3H/HeJ mice as well as in MDP-primed C3H/HeN mice. In contrast to the high cytokine-inducing activities of p-TUA in MDP-primed C3H/HeN mice (Fig. 1), in MDP-primed C3H/HeN mice the cytokine-inducing activities of p-TUA were weak; no TNF- α - and only low IL-6-inducing activities were shown. p-TUA-[H] induced practically no cytokines in this respect (Fig. 5). Furthermore, p-TUA and p-TUA-[H] showed no TNF- α - or IL-6-inducing activities in peritoneal macrophage cultures from C3H/HeJ mice.

Cytokine-inducing activities of teichuronic acids in human whole-blood cultures

p-TUA induced TNF- α and IL-6 in human whole-blood cultures, but p-TUA-[H] did not (Fig. 6).

Discussion

This is the first report to demonstrate that teichuronic acids from *M. luteus* induced inflammatory cytokines, TNF- α and IL-6, *in vivo* and *in vitro*. Whole bacterial

cells subjected to mild acid treatment and mild Smith degradation to remove teichuronic acids induced no TNF- α and little IL-6 in MDP-primed C3H/HeN mice. Whole cells lacking teichuronic acids (strains AH-47 and AH-149) induced no TNF- α and little IL-6 [12]. These findings support the concept that teichuronic acids are responsible for the cytokine induction.

Peptidoglycans showed no immunostimulatory activities in murine macrophage cultures [21], and those of *M. luteus* were reported to show only weak immunoadjuvant activity in guinea-pigs as compared with other bacterial peptidoglycans [19, 20]. In the present study, no induction of anaphylactoid reactions was observed in MDP-primed and peptidoglycan-challenged C3H/HeN mice and little or no TNF- α or IL-6 was detected in the serum of the mice. It was shown by Raman laser spectroscopy that peptidoglycans from IFO 3333 monococcal cells, MT tetrad cells and MT/T packet cells differed in the conformation of their glycan chains [33]. However, no difference was observed in their ability to induce anaphylactoid reactions and serum cytokines in MDP-primed C3H/HeN mice in the present study.

Teichuronic acids are polymers in which *N*-acetylmannosaminuronic acid and glucose residues are alternatively linked (see *Materials and methods*, p-TUA). The ability of the teichuronic acid preparations to induce cytokines in MDP-primed C3H/HeN mice varied with the bacterial strains from which they had been prepared. The reason why TUA (B 361, SCW), TUA (NCTC 2665, SCW) and p-TUA, i.e., teichuronic acids from the strains B 361 and NCTC 2665 had stronger cytokine-inducing ability than TUA from other cells is not known. It should be noted that the teichuronic acids

Fig. 4. Induction of cytokines by teichuronic acids in peritoneal macrophage cultures from C3H/HeN mice. Thioglycollate medium-induced peritoneal macrophages ($10^5/100 \mu\text{l}$ /well of a flat-bottomed microculture plate) of C3H/HeN mice were cultured in RPMI 1640 medium with or without test materials at 37°C for 24 h. The TNF- α and IL-6 activities of the culture supernate are shown in the left and right columns, respectively. The concentrations of samples are $\mu\text{g/ml}$.

Fig. 5. Near failure of teichuronic acids to induce cytokines in MDP-primed C3H/HeJ mice. Procedures were the same as those described in Fig. 1, except for the use of C3H/HeJ mice instead of C3H/HeN mice. TNF- α level is shown in upper bars and IL-6 level in lower bars, in MDP-primed C3H/HeJ mice (left column) and in MDP-primed C3H/HeN mice (right column; for comparison, data in Fig. 1 are reprinted). In MDP-primed C3H/HeJ mice only low IL-6 level was detected as shown in left column.

Fig. 6. Induction of cytokines by teichuronic acids in human whole-blood cultures. Heparinised peripheral blood ($25 \mu\text{l}$) was directly (without fractionation) incubated with test materials at the indicated doses in RPMI 1640 medium ($100 \mu\text{l}$) at 37°C for 24 h. The TNF- α and IL-6 levels in the culture supernate are shown in the left and right columns, respectively. The concentrations of samples are $\mu\text{g/ml}$.

prepared from cell walls lacking cytokine-inducing activities in the previous study [12] had practically no cytokine-inducing activities. Furthermore, IFO 3333, MT, MT/T and NCTC 2665 cells aggregated with the antiserum containing anti-teichuronic acid antibody [34], and mild acid treatment of these cells decreased their agglutinability with the serum. Cell walls from

these strains contain hexose corresponding to glucose of teichuronic acids, and mild acid treatment of these cell walls decreased the hexose content [25–27]. Teichuronic acids from B 361, IFO 3333, MT, MT/T and NCTC 2665 cells gave precipitin lines which fused with the line of p-TUA in an immunodiffusion experiment (unpublished data). The presence of three

kinds of chemical structures for the linkage region of teichuronic acids and peptidoglycans is suggested [35–38]. Although there is no evidence available, a fine or additional structure besides the main backbone consisting of a *N*-acetylmannosaminuronic acid and glucose may be different among these teichuronic acids.

Reduced teichuronic acids, polymers in which *N*-acetylmannosamine and glucose residues are alternately linked (see *Materials and methods*, p-TUA-[H]), did not induce cytokines. This may indicate that the carboxyl groups of the *N*-acetylmannosaminuronic acid residues play an important role in the induction of cytokines. Therefore, the effect of the addition of poly-L-lysine and poly-L-glutamic acid on the ability of teichuronic acids to induce cytokines in MDP-primed C3H/HeN mice was examined. Poly-L-lysine showed an inhibitory effect of p-TUA on cytokine-inducing activities, presumably due to an electrostatic interaction between positively charged amino groups of poly-L-lysine and negatively charged carboxyl groups of p-TUA, or the cytotoxic effect of poly-L-lysine, or both [39, 40]. Peptidoglycans, glycans, poly-L-glutamic acid and MDP suppressed the TNF- α induction by p-TUA, probably due to the competitive inhibition by their carboxyl groups on those of p-TUA. Although peptidoglycans and glycans significantly inhibited the IL-6 induction by p-TUA, poly-L-glutamic acid and MDP enhanced the IL-6 induction by p-TUA. Further study is necessary to elucidate the suppression of TNF- α inducing activity and the enhancement of IL-6-inducing activity of p-TUA by poly-L-glutamic acid and MDP.

It is well known that C3H/HeJ mice are resistant to LPS. Recently, a genetic reason for the unresponsiveness of these mice to LPS was revealed; C3H/HeJ mice have a point mutation in Toll-like receptor 4 (TLR4) [41–43]. As expected, TLR4-deficient (TLR4^{-/-}) mice did not respond to LPS [41]. In this context, a recent study by Takeuchi *et al.* [44] showed that purified lipoteichoic acids (LTA) from *Staphylococcus aureus* and *Streptococcus sanguis* induced TNF- α and IL-6 in peritoneal macrophage cultures from TLR4^{+/+} but not TLR4^{-/-} mice. LTA are the amphiphilic materials associated with the cytoplasmic membrane of most gram-positive bacteria except *M. luteus* [45, 46]. In this study, the cytokine-inducing activities of p-TUA, which is almost free from LPS, exhibited little or no activity both in *in vivo* and *in vitro* in C3H/HeJ mice. These findings suggest that not only LPS but also other bacterial cell surface components are recognised by TLR4 to activate the innate immunity system.

p-TUA also induced TNF- α and IL-6 in human peripheral blood cultures. The present study has shown that the teichuronic acid moiety was the only active component of *M. luteus* cell walls capable of inducing inflammatory cytokines *in vivo* and *in vitro*. Therefore, teichuronic acids might be responsible for the inflam-

matory diseases induced by *M. luteus*. However, teichuronic acids did not induce anaphylactoid reactions in the MDP-primed C3H/HeN mice, whereas whole cells containing low levels of teichuronic acids following treatment and whole cells lacking teichuronic acids (mutant strains AH-47 and AH-149) did [12]. Also, peptidoglycans did not induce anaphylactoid reactions in MDP-primed C3H/HeN mice. A recent study by Shibazaki *et al.* [47] revealed that complement activation followed by platelet degradation is involved in anaphylactoid reactions induced by LPS. These findings suggest that *M. luteus* cell surface materials other than teichuronic acids and peptidoglycans, which powerfully activate the complement system, might induce anaphylactoid reactions. In this context, poly-L-lysine, which induced an anaphylactoid reaction (Fig. 3), activates the complement system *via* C-reactive protein [48].

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