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Purification and characterization of a novel superantigen produced by a clinical isolate of *Yersinia pseudotuberculosis*

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Abstract A superantigen designated as *Yersinia pseudotuberculosis*-derived mitogen (YPM) was purified in an equal manner from both the culture supernatant and cell lysate of a clinical isolate (KUR-1) of *Y. pseudotuberculosis* serotype 4b. A significant proliferative response of human peripheral blood mononuclear cells to purified YPM was detectable even at a concentration of 1 pg/ml. The N-terminal sequence of YPM which included 23 amino acid residues was determined, by automated Edman degradation, as Thr-Asp-Tyr-Asp-Asn-Thr-Leu-Asn-Ser-Ile-Pro-Ser-Leu-Arg-Ile-Pro-Asn-Il e-Ala-Thr-Tyr-Thr-Gly-. This sequence differed from not only all the, hitherto, reported superantigens but also known proteins. While molecular weights of known bacterial superantigens are more than 22,000, electrospray ionization mass spectrometry showed that the molecular weight of YPM was 14524.4. These results indicate that YPM comprises a novel superantigen with substantial structural differences from other bacterial superantigens produced by Gram-positive cocci.

Key words: Superantigen; Purification; Mitogen; N-terminal sequence; ESI-MS; *Yersinia pseudotuberculosis*

1. Introduction

Superantigens are highly potent immunostimulatory molecules that bind, as an intact protein, to outside of the conventional peptide antigen-binding site of a class II major histocompatibility complex molecule, and are capable of activating virtually all T-cells that express particular β chain variable region (V_β) of T-cell receptor (TCR) [1–4]. Members of the superantigen family include viral superantigens such as the minor lymphocyte stimulatory antigens from mouse mammary tumour virus and bacterial toxins produced by *Staphylococcus aureus* and other bacteria. The toxins with superantigenic activity have been directly implicated in acute and systemic diseases in human. The toxicity of bacterial superantigens is thought to be mediated by their potent T-cell-stimulating activities, leading to lymphokine release.

Yersinia pseudotuberculosis is a Gram-negative enteropathogenic bacillus which induces not only gastrointestinal symptoms but also a variety of systemic symptoms such as fever, scarlatiniform rash, mesenteric lymphadenitis, acute renal failure, and arthritis [5–7]. The clinical pathophysiology of *Y. pseudotuberculosis* infection has many overlapping symptoms caused by *S. aureus* and *Streptococcus pyogenes* which produce a superantigen. Recently, Uchiyama et al. [8,9] and we [10] have independently demonstrated the production of a superantigen by *Y. pseudotuberculosis*, and designated the superantigen as *Y. pseudotuberculosis*-derived mitogen (YPM). While the biological property of YPM was superantigenic, YPM was different from the known bacterial superantigens with respect to a TCR V_β repertoire expressed in selectively activated T-cell populations [8,10]. Furthermore, it has been reported that the T-cell response to YPM was not neutralized by antibodies against known bacterial superantigens, toxic shock syndrome toxin-1 (TSST-1) and five types of staphylococcal enterotoxin (SE), produced by *S. aureus* [10]. These facts implied the possibility that YPM is a novel bacterial superantigen with large structural differences from the known bacterial superantigens. This formed the impetus for the present study in which we report the purification, N-terminal sequence, and molecular weight of a novel bacterial superantigen produced by *Y. pseudotuberculosis*.

2. Experimental

2.1. Bacterial strain and culture conditions

A clinical strain of *Y. pseudotuberculosis* (KUR-1) belonging to the serotype 4b isolated from a patient manifesting Kawasaki disease-like symptom was donated by Dr. N. Takeda (Kurashiki Central Hospital, Kurashiki, Japan). The strain was cultured as described previously [10].

2.2. Purification of YPM from the culture supernatant

The culture supernatant of *Y. pseudotuberculosis* KUR-1 was precipitated with 80% ammonium sulfate. After centrifugation at 15,000 × *g* for 30 min at 4°C, the resulting pellet was resuspended in 10 mM sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer. Dialyzed samples were subjected to gel filtration on a Sephacryl S-200 Superfine column (3 × 100 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0), and eluted with the same buffer at a flow rate of 33 ml/h. An aliquot of each fraction collected in 10 ml was tested for proliferative response of human peripheral blood mononuclear cells (PBMC). The mitogenic fractions were concentrated, dialyzed against 20mM Tris-HCl buffer (pH 7.8), and then purified further by high-performance ion-exchange chromatography (HPIEC) and reversed-phase high-performance liquid chromatography (RP-HPLC).

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Abbreviations: ESI-MS, electrospray ionization mass spectrometry; HPIEC, high-performance ion-exchange chromatography; PBMC, peripheral blood mononuclear cells; RP-HPLC, reversed-phase high-performance liquid chromatography; SE, staphylococcal enterotoxin; TCR, T-cell receptor; TFA, trifluoroacetic acid; TSST-1, toxic shock syndrome toxin-1; YPM, *Yersinia pseudotuberculosis*-derived mitogen; V_β, β chain variable region.

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2.3. Purification of YPM from the cell lysate

Cells of *Y. pseudotuberculosis* KUR-1 collected by centrifugation were suspended in 20 mM Tris-HCl buffer (pH 7.2), disrupted by sonication, and centrifuged at 10,000 × *g* for 20 min at 4°C. After ultracentrifugation of the supernatant at 100,000 × *g* for 3 h at 4°C, the resulting supernatant was treated with ammonium sulfate (80% saturation). The precipitates were resuspended, dialyzed against the same buffer, and purified further by HPIEC and RP-HPLC.

2.4. HPIEC

A sample solution was applied to a TSKgel DEAE-5PW column (7.5 × 75 mm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.8) and eluted with the equilibration buffer for 5 min, followed by a linear gradient of NaCl (0–0.3 M in 25 min) in the same buffer, at a flow rate of 1.0 ml/min.

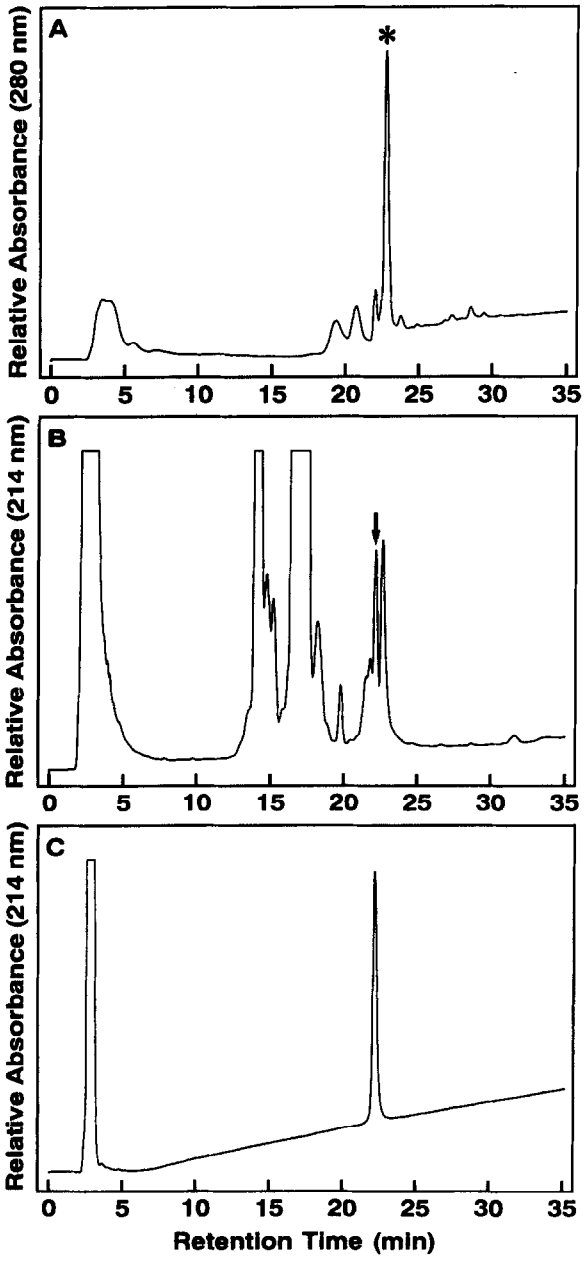

Fig. 1. Purification of YPM. (A) HPIEC of the crude YPM obtained by Sephacryl S-200 Superfine gel filtration. (B) RP-HPLC of the mitogenic peak fraction indicated by an asterisk in (A). An arrow indicates the peak fraction exhibiting potent mitogenic activity. (C) Rechromatography RP-HPLC of the purified preparation of YPM.

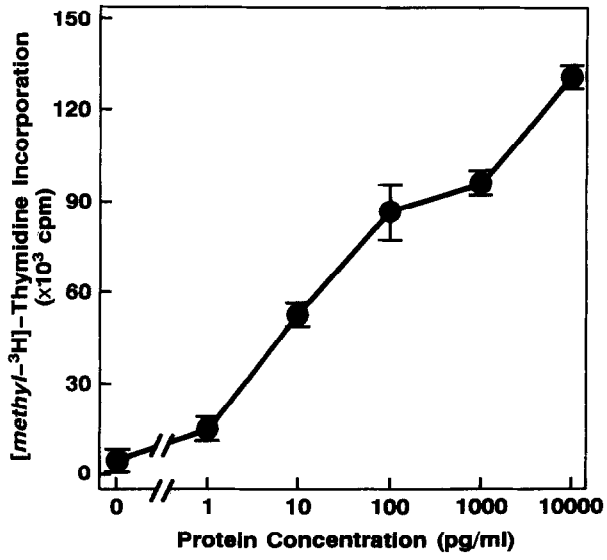

Fig. 2. Dose–response curve of human PBMC proliferation stimulated by the purified YPM. Values are the mean of three determinants ± S.E.M. Protein concentration was determined by RP-HPLC using ribonuclease A with a molecular weight of 13,682 as the standard.

2.5. RP-HPLC

RP-HPLC for the separation of YPM was carried out using a Develosil 300C4-HG-5 column (4.6 × 150 mm) previously equilibrated with 30% acetonitrile in 0.1% trifluoroacetic acid (TFA) and run with a linear gradient of acetonitrile (30–40% in 30 min), at a flow rate of 1.0 ml/min.

2.6. Electrospray ionization mass spectrometry (ESI-MS)

ESI mass spectrum was measured by a JEOL JMS-HX/HX110A four-sector tandem mass spectrometer (Tokyo, Japan) equipped with an ESI ion source (Analytica of Branford, Branford, CT). The sample was dissolved in an equal volume of mixture of acetonitrile/0.2% TFA in water/2-methoxyethanol/2-propanol and delivered at a flow rate of 1 μl/min.

2.7. Peptide synthesis

Chemical synthesis of two peptides NP-I (CTDYDNTLNSIPSLRI) and NP-II (CLNSIPSLRIPNIATY) were performed using the continuous flow solid-phase technique with use of a MilliGen/Biosearch 9050 peptide synthesizer (Bedford, MA). The peptide chains were elongated stepwise on polyethylene glycol polystyrene resin, using optimized 9-fluorenylmethoxycarbonyl chemistry, as previously described [11–13]. The synthetic peptides were purified by RP-HPLC and characterized by amino acid analysis and fast atom bombardment mass spectrometry, giving the expected values.

2.8. Antisera preparation

The synthetic peptide with sulfhydryl group (2 mg) in 200 μl of a conjugation buffer (83 mM sodium phosphate, 150 mM NaCl, 0.1 M EDTA, 0.02% NaN3, pH 7.2) was mixed with a solution of the carrier protein (bovine serum albumin, 2 mg in 200 μl of the conjugation buffer), which was previously cationized to an optimum level and activated with sulfhydryl reactive maleimide groups [14,15]. The reaction mixture was incubated for 2 h at room temperature and then was dialyzed against a buffer (83 mM sodium phosphate, 150 mM NaCl, pH 7.2).

Rabbits were immunized with the peptide–carrier protein conjugate (0.1 mg) emulsified with Freund complete adjuvant by injecting into each of ten subcutaneous sites on the back. After 3 weeks, the rabbits were boosted with the conjugates in Freund complete/incomplete adjuvant. Antisera were obtained by bleeding the rabbits one week after the booster and were tested for antibody response by enzyme-linked immunosorbent assay.

Rabbit antiserum against YPM was raised by repeated immunizations (3 times at 4-week intervals) with ca. 25 μg of the purified prepa-

ration. Antiserum obtained 10 days after the last booster was tested for mitogenic activity-neutralizing response.

2.9. Assay for proliferative response

Proliferative response was measured with human PBMC cultures as described previously [10]. For neutralization experiments, PBMC cultures were incubated with the purified YPM (ca. 10 pg/ml) in the presence or absence of each antiserum (10 μl).

3. Results

3.1. Purification of YPM from the culture supernatant

Crude YPM obtained from the culture supernatant of *Y. pseudotuberculosis* 4b strain KUR-1 was partially purified by ammonium sulfate precipitation and gel filtration, as described in section 2. The mitogenic fractions on human PBMC were purified further by HPIEC. One major peak and several minor peaks were observed in the elution profile (Fig. 1A), and mitogenic activity was eluted only in the major peak fraction. This fraction was subjected to RP-HPLC to give a pure mitogen and the fraction which showed potent mitogenic activity was eluted as one of the quite minor peaks to yield pure YPM (Fig. 1B). Proliferative response of human PBMC to purified YPM was detectable even at a concentration of 1 pg/ml (Fig. 2). The fraction with marked mitogenicity was subjected to rechromatography to give a sharp and single peak, indicating purification to high homegeneity (Fig. 1C). Furthermore, rabbit antiserum against the purified preparation completely neutralized the YPM-induced proliferation (Fig. 3). This also showed that the YPM preparation obtained here was highly homogeneous.

3.2. The N-terminal amino acid sequence and molecular weight of YPM

Automated Edman degradation of purified YPM, using a Shimadzu PSQ-1 gas-phase protein sequencer (Kyoto, Japan), provided the N-terminal sequence of 23 amino acid residues as Thr-Asp-Tyr-Asp-Asn-Thr-Leu-Asn-Ser-Ile-Pro-Ser-Leu-Arg-Ile-Pro-Asn-Il e-Ala-Thr-Tyr-Thr-Gly-. For confirmation that the determined N-terminal sequence is certainly that of YPM,

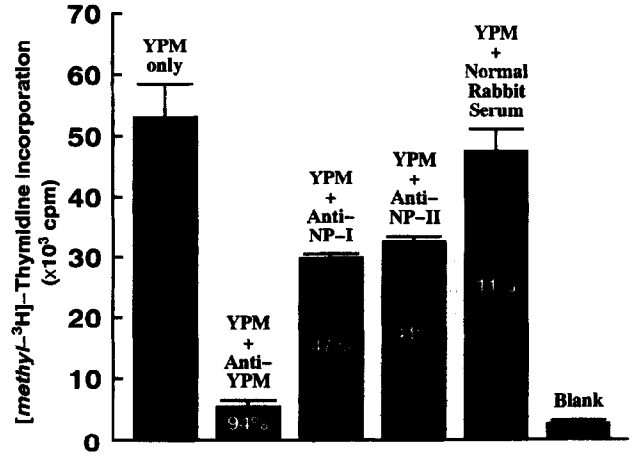

Fig. 3. Inhibition of the YPM-induced proliferation by antisera against purified YPM and against the synthetic peptides NP-I and NP-II. Values are the mean of three determinants ± S.E.M. The number in a column denotes the percentage of inhibition, which was calculated as the ratio of the difference between the observed and blank cpm values to that between maximum (obtained in the incubation with only YPM) and blank cpm values.

rabbit antisera against synthetic peptides NP-I and NP-II were raised and their neutralizing capabilities were tested. Peptides NP-I and NP-II represent partial sequences Thr^{1}–Ile^{15} and Leu^{7}–Tyr^{21} of the determined sequence, respectively, and possess a cysteine residue at the N-terminus for conjugation with the maleimide-activated carrier protein. As shown in Fig. 3, the proliferative response of human PBMC to YPM was partially inhibited by antisera against NP-I and NP-II.

In the ESI mass spectrum of purified YPM, four intense signals were observed (Fig. 4). From the observed values of these signals, the molecular weight of the YPM was calculated to be 14,524.4 ± 0.3.

3.3. Purification of YPM from cell lysate

Since considerable mitogenic activity was detected in the cell lysate, we purified the mitogen from the cell lysate to compare with YPM obtained from the culture supernatants. The cell lysate of *Y. pseudotuberculosis* strain KUR-1, obtained as described in section 2, was purified by ammonium sulfate precipitation, HPIEC and by RP-HPLC using the same conditions as in the purification from the culture supernatant. At each step, potent mitogenic activity was eluted in a peak fraction with nearly identical retention time to that of the active peak fraction shown in Fig. 1 (data not shown). The N-terminal 15 amino acid residues of the purified mitogen were determined to be identical to that from the culture supernatant. ESI-MS of the purified preparation from the cell lysate showed that the molecular weight was 14,524.2 ± 0.1. These results indicate that YPM preparation from both the supernatant and the cell lysate are the same molecules.

4. Discussion

In the present study, a superantigen designated YPM was purified to homogeneity from both the culture supernatant and cell lysate of a clinical isolate of *Y. pseudotuberculosis*, and its N-terminal amino acid sequence and molecular weight were determined. Although Gram-positive coccal superantigens, such as TSST-1, SEs and streptococcal pyrogenic exotoxins, are well characterized, the present study concerned the purification and characterization of a bacterial superantigen produced by a Gram-negative bacillus.

While the amino acid sequences of various superantigens produced by Gram-positive cocci are known [16–28], neither the N-terminal sequence nor the sequence of other regions of these superantigens were identical to the N-terminal sequence of the purified YPM determined in this study. Furthermore, the sequence with 23 amino acid residues was not notably homologous to any sequence of previously characterized proteins. Although molecular sizes of the previously documented bacterial superantigens range from 22,000 to 29,000, ESI-MS of the purified YPM showed its molecular weight to be 14,524.4. From these results based on the protein chemistry, it is concluded that YPM is a novel superantigen whose molecular structure is quite different from Gram-positive coccal superantigens.

The present study showed that the mitogenic substances present in both the culture supernatant and the cell lysate are identical. This implied that YPM is synthesized in the cell as the active form and is secreted as such extracellularly without any further processing.

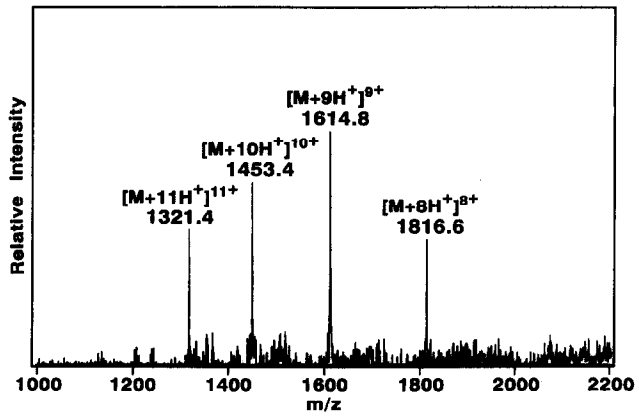

Fig. 4. ESI mass spectrum of the purified YPM. From each observed m/z value of four multiply charged ion signals ($[M + nH]^{n+}$), molecular weight of purified YPM was calculated to be 14,524.4 ± 0.3.

Braun et al. [29] have cautioned that the mitogenicity on T-cells could be due to similar minute contaminations with potent superantigens that are undetectable by current biochemical methods but are, nonetheless, extremely effective in stimulating sensitive T-cells. Consequently, an argument can be raised that the N-terminal sequence and molecular weight determined in this study are certainly the character of YPM. We confirmed this by demonstrating that synthetic peptides harboring the N-terminal sequence determined here could induced a neutralizing antibody for the human PBMC proliferation stimulated by YPM.

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