Isolation and Characterization of a Polysaccharide Antigen from *Propionibacterium acnes* Released by a Glycine-Specific Chemical Protein Degradation Procedure

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Dedicated to Professor Wilhelm Menke on the occasion of his 80th birthday

P. acnes Polysaccharide, Mercuric Oxycyanide, Glycyl-Peptide Bond Cleavage, Carbohydrate Composition, Antigenic Titer

An acid-labile antigenic polysaccharide has been isolated from both cell walls and culture media of *Propionibacterium acnes* using a new chemical degradation procedure which liberates protein-bound or associated carbohydrate. Lyophilized cells and culture media were treated with a suspension of mercuric oxide in a solution of alkaline mercuric cyanide for several hours at room temperature liberating water-soluble polysaccharide material. The antigenic polysaccharide was freed of reaction products by alcohol extraction and purified by anion exchange chromatography and gel filtration, resulting in three distinct fractions of acidic polysaccharides of apparent molecular weights between 15–150 kDa. Sugar analysis showed the polysaccharides to contain fucose, galactose, glucose, mannose, galactosamine, glucosamine, and 2,3-diamino-2,3-dideoxy-D-glucuronic acid. The three fractions also contained amino acids, predominantly glutamic acid, alanine, and glycine, known to be components of *P. acnes* cell wall peptidoglycan. All three molecular weight fractions reacted with rabbit antisera raised against whole *P. acnes* cells, with the highest titer for both cell and media-derived polysaccharide material consistently in the high molecular weight fraction. This procedure was also capable of releasing antigenic polysaccharide from tissues of rats administered *P. acnes* cells or radiolabeled cell wall fragments.

Introduction

Propionibacterium acnes (Corynebacterium parvum, Corynebacterium acnes) has been extensively studied in an attempt to elucidate the nature of the biologically active component(s) of its cell wall. This bacterium is capable of activating complement by both classical and alternate pathways [1, 2], stimulating the reticuloendothelial system [3–7], macrophage activation [8–11], and antitumor activities [12–14]. These studies have demonstrated that both polysaccharide and peptidoglycan play an important role in the biological activity of *P. acnes*. Furthermore, a phenol-water extractable antigen immunologically identical to a *P. acnes* antigen has been isolated from synovial fluid and synovial fluid leukocytes of patients with rheumatoid arthritis [15, 16].

Methods for the isolation, purification, and biochemical characterization of acidic polysaccharides from *P. acnes* culture media have involved the use of pronase digestion followed by ethanol precipitation [17], of ethanol extraction alone [18], or of phenol-water extraction followed by proteolysis with pronase [19]. Acidic polysaccharide has been extracted from *P. acnes* cell walls with urea [18], phenol-water followed by proteolysis with pronase [19], hot 5% trichloroacetic acid [20], hot 0.5 N sodium hydroxide solution [21] or by proteolysis with lysozyme followed by mild acid treatment with 50 mM glycine-HCl [22]. These methods are typically lengthy, and can result in loss of acid-labile sugars during hot TCA or formamide extraction.

We report here on the high-yield isolation of acidic polysaccharide material from both *P. acnes* cells and culture media using a new chemical deg-

Abbreviations: OPA, orthophthalaldehyde; DiNAcGlcUA, 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid; DAGlcUA, 2,3-diamino-2,3-dideoxy-D-glucuronic acid; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; CIE, counter-immunoelectrophoresis; BSA, bovine serum albumin; TFA, trifluoroacetic acid.

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radation procedure which rapidly solubilizes bacterial cells and liberates protein-bound or associated carbohydrate intact from a variety of materials. The procedure is based upon the oxidation of glycyl residues to oxalate in the presence of mercuric oxide (mercuric oxycyanide) with concomitant peptide bond cleavage [23] but preservation of carbohydrate structure [24]. This procedure has also been used to digest tissue from rats administered *P. acnes* cells or radiolabeled cell wall fragments. The titer of the released antigenic material correlated with the amount of radioactivity recovered.

Materials and Methods

Materials

2-Cyanoacetamide and orthophthalaldehyde (OPA) were obtained commercially of available grade. Amino acid calibration standard (Standard H) was obtained from Pierce, hexosamines were obtained as reagent-grade hydrochloric acid salts, while neutral sugars and all other chemicals were of the highest grade commercially available. Rabbit polyclonal antisera to *P. acnes* whole cells were generated according to reference [19]. 2,3-Diacetamido-2,3-dideoxy-D-glucuronic acid was synthesized according to Cummins and White [20] from 2,3-diacetamido-2,3-dideoxy-D-glucose (U.S. Biochemical Corp.), and purified and analyzed as previously described [25]. Specific pathogen-free Lewis/N female rats 4–5 weeks of age (100 g) were obtained from Harlan Sprague Dawley, Inc.

Bacterial strain and cultivation

Propionibacterium acnes ATCC 6922, obtained from the American Type Culture Collection, Rockville, Md., was grown in 2.5 l batches in 4 l Erlenmeyer flasks at 37 °C for 3–5 days in brain heart infusion media (BBL Microbiology Systems). Cultures were heat killed (70 °C, 2 h) and the cells harvested by centrifugation (8000 × *g*, 25 °C, 20 min) followed by two washes with distilled water. Packed cells were lyophilized in a 500 ml round bottom flask and stored at 25 °C. Culture media and water washes were pooled, dialyzed, concentrated, and lyophilized. The bacteria were grown on [^{14}C-U]glucose (ICN) to obtain radiolabeled cells and soluble cell wall polysaccharide antigen.

Assay of soluble antigen

P. acnes antigen was identified and quantitated immunologically using counterimmunoelectrophoresis (CIE) as previously described [19]. Typically, samples at a concentration of 1 mg/ml (1:1) were diluted (serially or tenfold) and assayed against polyclonal rabbit *anti-P. acnes*.

Large-scale preparation of acidic polysaccharide antigen

Whole cells and culture media were digested using the alkaline mercuric oxycyanide procedure [24, 26]. All steps were performed at room temperature (25 °C) unless otherwise noted. Briefly, 5 g solid HgO and 50 ml of a 20% solution of mercuric cyanide in 2 M potassium hydroxide were added to 5 g lyophilized bacterial material in a 500 ml round bottom flask. The solution was magnetically stirred for 6 h, and then the flask was placed in an ice bucket under a hood and the reaction terminated by acidification to pH 5 with glacial acetic acid. Organic and inorganic mercurials were reduced to mercury metal by the addition of 4.2 ml borane-pyridine complex. After the generation of HCN was complete, the reaction mixture was dried by rotary evaporation or lyophilization. 95% ethanol (250 ml) was added to the dry black residue which was resuspended at 45 °C by sonication in a bath sonicator. The suspension was centrifuged (10,000 × *g*, 30 min) and the mercury-free supernatant containing mostly lipids and potassium acetate decanted. The pellet was resuspended in 10% aqueous pyridine (250 ml) and sonicated to dissolve soluble oligo- and polysaccharides as well as low molecular weight peptide degradation products. The suspension was centrifuged (10,000 × *g*, 30 min) and the clear supernatant decanted off of the mercury pellet and lyophilized. The resulting material was resuspended in 10% pyridine and desalted on a column (5 × 95 cm; 2.5 × 70 cm) of Sephadex G25 (Pharmacia). The carbohydrate-containing fractions were pooled, concentrated, and applied to a column (2.5 × 39 cm) of QAE-Tris-Acryl (LKB/Pharmacia) equilibrated with 0.22 M NH_4OH in 2.5% *n*-butanol and eluted at 65 ml/h with a 0–3 M concave gradient of ammonium acetate, pH 6.

Fractions (13 ml) were assayed for total hexose and antigenic titer by CIE to determine those fractions which contained antigenic polysaccharide. Positive fractions were pooled, concentrated, and applied to a column (1.8 × 98 cm; 2.5 × 83 cm) of Sepharose CL-6B (Pharmacia) equilibrated and eluted with 2.5% *n*-butanol in water. Fractions (7 ml) were pooled as above.

Isolation of P. acnes antigen from rat tissues

Lewis/N female rats (four) were administered 25 mg heat-killed *P. acnes* cells in 3 ml sterile 0.9% sodium chloride by intraperitoneal (i.p.) injection on day 0, sacrificed on day 7, and like tissues combined for digestion using a modified alkaline mercuric oxycyanide procedure. Briefly, 0.2 g solid HgO and 1 ml of a 20% solution of mercuric cyanide in 2 M potassium hydroxide were added to 1 g tissue (wet weight) in a polypropylene centrifuge tube. Samples were tumbled for 6 h, and then the reactions were terminated by addition of 0.25 ml glacial acetic acid, followed by the addition of 0.2 ml borane-pyridine complex. Samples were dried at 65 °C in a vented oven, and then the resulting dry black residue was resuspended in 40 ml 95% ethanol, centrifuged, and the supernatant decanted. The pellet was resuspended in 20 ml distilled water and centrifuged. The supernatants were desalted (Bio-Rad Econo-Pak™10DG), lyophilized, resuspended in water to a concentration of 100 mg/ml, and tenfold dilutions analyzed by CIE for antigenic titer. Additionally, rats received an injection of bacterial cell wall fragments prepared by sodium dodecyl sulfate extraction and sonication as previously described [27]. On day 0, two rats received a single i.p. injection containing 32 mg 40,000 × *g* supernatant and 32 mg 40,000 × *g* pellet (8.3×10^6 cpm) in 3 ml sterile 0.9% sodium chloride. Rats were sacrificed after 32 days and their tissues treated as above. The water extracts were desalted (CentriCell Ultrafiltration – 10,000 mol. wt. limit, Polysciences Inc.), lyophilized, and resuspended in water to a concentration of 4 to 128 mg/ml depending on recovery. Aliquots were taken for liquid scintillation counting and for analysis by CIE of serial dilutions. Control animals (six) were sacrificed 42 days after receiving a single i.p. injection of sterile 0.9% sodium chloride (3 ml).

Analytical methods

Total hexose was estimated by the phenol-sulfuric acid method [28] with D-glucose as standard. Diaminoglucuronic acid was analyzed as previously described [25]. Total sugars were determined after hydrolysis with 4 M TFA (105 °C, 4 h) by anion exchange chromatography. The free sugars were separated simultaneously on a column (HPIC-AS6, Dionex Corp.) eluted at 30 ml/h with 10 mM sodium hydroxide (pH 12) with either pulsed amperometric [29, 30] or 2-cyanoacetamide [31, 32] detection. Amino sugars were determined after hydrolysis with 4 M TFA (105 °C, 4 h) by cation exchange chromatography on a column (0.9 × 18 cm, 50 °C) of DC-6A (Dionex) eluted at 70 ml/h with 0.35 M sodium citrate buffer, pH 5.28, containing 0.2 M boric acid with OPA detection [33]. Amino acids were determined after hydrolysis with 6 M HCl (105 °C, 24 h) on a Waters Pico-Tag column as PITC derivatives. Muramic acid was determined after hydrolysis with 6 M HCl (105 °C, 24 h) by cation exchange chromatography on a column (0.9 × 30 cm, 40 °C) of DC-6A (Dionex) eluted at 50 ml/h with Pierce pHix Pico Buffer A (System II) [34, 35] with OPA detection [33].

Results

Isolation and characterization of P. acnes polysaccharide antigens

The organisms were routinely grown in several 2.5 l batches yielding 0.5 g (dry weight)/l to generate the bacterial cells necessary for the extraction of polysaccharide material. Additionally, the organism releases a soluble, antigenically cross-reacting material derived from the cell walls into the culture media during its growth, which allowed monitoring of bacterial growth by assaying aliquots of the culture media for the released antigenic material by CIE against rabbit *anti-P. acnes*. Soluble antigenic material recovered by dialysis and concentration of the culture media after removal of the cells by centrifugation typically yielded 0.6 g (dry weight)/l of salt-free, high molecular weight material.

Treatment with the alkaline mercuric oxycyanide procedure yielded 1.9 g of white material from 15 g cells, and 8.6 g of tan material from 18 g dialyzed culture media. Lyophilized material from

both cells and culture media was further purified in 1 g aliquots by anion exchange chromatography. The break fraction, which contained neutral and cationic carbohydrate material but was negative for antigen, was well separated from a single antigen peak. Fractions positive for hexose and antigen were pooled, lyophilized, and resuspended in pyridine-water for gel filtration. ^{14}C-Labeled antigenic material from *P. acnes* culture media was separated by gel filtration into three antigenic peaks (Fig. 1). This profile is representative for the separations obtained during large-scale preparative runs. Material isolated from unlabeled media separated into three polydisperse antigenic peaks in the ranges of 175, 83 (maximum), and less than 15 kDa. Material from cells eluted as a broad high molecular weight band in the range of 100–200 kDa, which when rechromatographed, yielded two fractions in the ranges of 150–200 and 50–150 kDa. High and intermediate molecular weight fractions from both media and cells had the highest titers against rabbit *anti-P. acnes,* in contrast to the low molecular weight material (Table I).

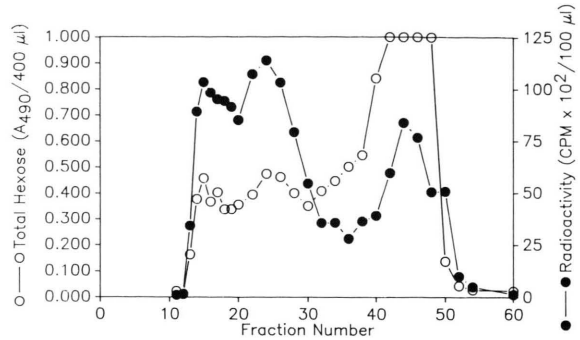

Fig. 1. Sepharose CL-6B (2.5 × 83 cm) gel filtration chromatography of antigenic polysaccharide isolated from ^{14}C-labeled *P. acnes* culture media. The profile reveals the heterogeneous nature of the antigenic material. Fractions 12–17 (I), 18–32 (II), and 33–59 (III) were pooled. Volume of each fraction, 10 ml. Aliquots were taken for phenol-sulfuric acid analysis and liquid scintillation counting. Molecular weight marker peaks eluted at fractions 18 (IgG), 30 (BSA), 38 (cytochrome *c*).

Purified polysaccharide was found to contain mainly neutral and amino sugars, with only trace amounts of uronic acids and ketoses detected by colorimetric assay (data not shown). Table II summarizes the sugar and amino acid composition of high molecular weight antigen purified from whole cells and culture media. The major neutral sugar components were determined to be galactose, glucose and mannose, while the major amino sugar components were glucosamine and galactosamine. High molecular weight antigen contained approximately 5% DiNAcGlcUA, while low molecular weight antigen contained trace amounts (data not

Table I. Purification of *P. acnes* polysaccharide from 15 g whole cells and 30 l culture media.

Fraction	Total hexose[a] [%]	Antigenic titer[b]	Sample weight [mg]	% Yield
Lyophilized whole cells	12	1: 4	15,000	100.0
Water extract	43	1: 512	1,941	12.9
Gel filtration				
high mol. wt.	39	1:2048	20	0.1
int. mol. wt.	33	1:1024	81	0.5
low mol. wt.	22	1: 512	952	6.3
Lyophilized culture media	–[c]	1: –	17,750	100.0
Water extract	16	1: 256	8,656	49.0
Gel filtration				
high mol. wt.	37	1:4096	415	2.3
int. mol. wt.	18	1: 512	1,013	5.7
low mol. wt.	4	1: 16	2,692	15.2

[a] Total hexose was estimated by the phenol-sulfuric acid method [28].
[b] Fractions were serially titrated (1:1 to 1:16,384) and analyzed against rabbit *anti-P. acnes* by CIE.
[c] –, not determined.

Table II. Amino sugars, diacetamidoglucuronic acid, neutral sugars and amino acids (nmol per mg fraction dry weight) in high molecular weight acidic polysaccharide antigen isolated from *P. acnes* whole cell and culture media.

Component	Whole cell	Culture media
Sugars		
GlcNAc[a]	65	179
GalNAc[a]	84	155
DiNAcGlcUA[a]	146	193
Galactose[b]	320	538
Glucose[b]	1080	434
Mannose[b]	268	502
Amino acids[c]		
Aspartic acid	26	86
Threonine	32	111
Serine	159	253
Glutamic acid	310	342
Proline	22	197
Glycine	197	201
Alanine	432	348
Cysteine	–[d]	–
Valine	272	274
Methionine	–	–
Isoleucine	52	42
Leucine	27	48
Tyrosine	38	–
Phenylalanine	83	92
Histidine	–	–
Lysine	15	32
Arginine	34	51

[a] Amino sugars were determined by cation exchange chromatography with OPA detection.
[b] Neutral sugars were determined by anion exchange chromatography with pulsed amperometric detection.
[c] Amino acids were determined as PITC derivatives on a Waters Pico-Tag column.
[d] –, absent.

shown). The carbohydrate content of high molecular weight polysaccharide antigen correlated with its high titer (1:4096) against rabbit *anti-P. acnes*, with titers as high as 1:16,384 observed in some polysaccharide preparations. Although chromatographic analysis for peptidoglycan content yielded a small amount of muramic acid (data not shown), the amino acid analysis suggested that this value could be higher, possibly due to incomplete hydrolysis of glucosaminyl-muramic acid [36]; the fractions were not assayed for diaminopimelic acid.

Graded hydrolysis of high molecular weight antigen from culture media was performed in order to establish a correlation between structure and antigenicity (Fig. 2), followed by analysis for carbohydrate composition and antigenic titer. No

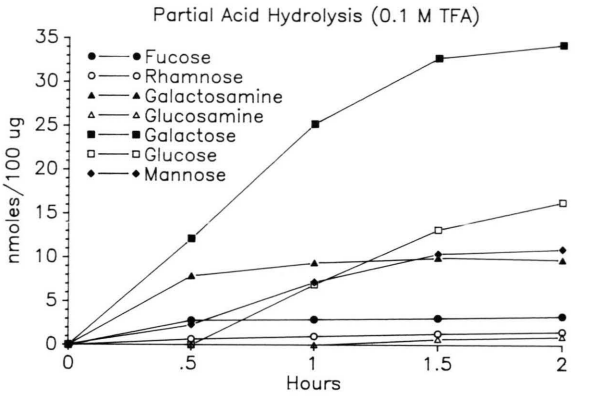

Fig. 2. Partial acid hydrolysis of high molecular weight antigen isolated from culture media. The sample was analyzed by anion exchange chromatography with pulsed amperometric detection after hydrolysis in 0.1 M trifluoroacetic acid at 105 °C for 30, 60, 90, und 120 min.

titer was detectable 30 min after partial acid hydrolysis although the starting material had a high antigenic titer (1:4096). This indicates the importance of terminal sugars as part of the antigenic site, which correlates most closely with the loss of acid-labile fucose and is further supported by the loss of antigenicity by periodate oxidation [37].

Amino acids constitute approximately 20% of the measured weight for high molecular weight antigen isolated from cells or culture media, while low molecular weight antigen contains a larger amount of peptide (60–70%) (data not shown). The decreased titer observed for low molecular weight fractions reinforces the concept of the importance of the saccharide moiety as part of the antigenic site. Polysaccharide from cells and media (Table II) contain predominantly those amino acids found in *P. acnes* peptidoglycan (glutamic acid, glycine, alanine) [36, 38], and the molar ratios of the amino acids isolated from either cells or media were similar, suggesting shedding of the antigen into the media.

Recovery of labeled antigen from rat tissues

When crude antigen with a titer of 1:1028 was added to homogenized rat liver and subsequently treated with the alkaline mercuric oxycyanide procedure 100% of the antigenic titer was recovered in the water-soluble extract (data not shown). When tissues from rats administered 25 mg *P. acnes* cells

were analyzed 1 week after injection, antigenic material was recovered from the spleen (1:10), liver (1:1), lung (1:1), and kidney (10:1); trace amounts were detected in front and hind paws. Pooled tissues from the six rats given saline were negative for antigen by CIE at a concentration of 10 mg/ml (10:1). Similarly, when tissues from two rats administered radiolabeled cell wall fragments were analyzed 32 days after injection, 6.4 and 10.5%, respectively, of the total administered radioactivity was recovered in water extracts of the digested tissues. The largest amounts of radioactivity were measured in the liver (63.0 and 83.4% of recovered counts, respectively) and spleen (33.9 and 13.1% of recovered counts, respectively), with trace amounts (0.1–2.4%) recovered in the kidney, lung, and the front and hind paws. For each tissue extract, the amount of radioactivity recovered appeared to be correlated with the strength of the antigenic titer. Indeed, analysis of the data gave a Pearson's correlation coefficient of $r = 0.91$ ($r^2 = 83\%$), which is considered to represent a fairly strong linear relationship.

Discussion

The acidic polysaccharides that have been isolated from both *P. acnes* cells and culture media by alkaline mercuric oxycyanide extraction resemble in size and composition acidic polysaccharides previously extracted by phenol-water [19], mild acid [17], and proteolysis [22], and the antigenic titer of the acidic polysaccharides correlates with size as shown (Table I). The immunoprecipitation of *P. acnes* polysaccharide by antibodies to *P. acnes* cells has been reported [17, 18] and used to track the material during purification. The acid lability of the titer correlates with the release of fucose from the polysaccharide. Treatment of antigenic material isolated by the alkaline mercuric oxycyanide procedure with either hot formamide or mild TCA resulted in a dramatic loss in titer, to less than 1% (data not shown). These reagents have been published for the isolation of polysaccharide material.

The amino acid and carbohydrate composition of high molecular weight acidic polysaccharide antigen agrees with published data. Dawes *et al.* [17] have described a high molecular weight antigen isolated from culture media containing approximately 40% carbohydrate and 10% protein, and having as major amino acids alanine, glutamic acid, and glycine which are characteristic for *P. acnes* cell wall peptidoglycan [36, 38]. Protein accounted for 20% of the measured weight of polysaccharide isolated by the alkaline mercuric oxycyanide extraction procedure, and the molar ratios we obtained are similar. The carbohydrate content reported [17] was mainly neutral (19%) and amino (14%) sugars. High molecular weight antigenic material isolated in our laboratory contains 30% neutral sugars and 10% amino sugars, 5% of which is DiNAcGlcUA. This unusual acidic amino sugar has been identified by GLC-MS [20] and ^{13}C NMR [39] in *P. acnes* cell wall polysaccharide, together with its 2-epimer diaminomannuronic acid [22]. The latter being possibly derived from DAGlcUA by epimerization as similarly observed for diaminomannose epimerization during acid hydrolysis of diaminoglucose [40]. We have occasionally observed in our hydrolysates of purified acidic polysaccharide antigen an unknown peak eluting between DAGlcUA and histidine by cation exchange chromatography which appears to represent an amount of material equal to DAGlcUA.

Propionibacterium acnes has been studied due to an interest in the biological activities of the cell wall components of this gram-positive anaerobic diptheroid. While others have addressed the tissue distribution of radiolabeled *P. acnes* in mice [13, 41, 42], attempts have not been made to recover polysaccharide antigen from such tissue. We report here the recovery of antigen from rats given ^{14}C-labeled bacterial cell wall fragments. It has been suggested that the occurrence of free amino groups on glucosamine in *P. acnes* peptidoglycan [36], which confers lysozyme resistance, is partly responsible for the long-term survival of cell wall polysaccharide during catabolism in mice [13, 41, 42] and isolated macrophages [43] or neutrophils and monocytes [44]. Although the conditions used to administer cells and radiolabeled cell wall fragments did not induce inflammation in any of the rats tested, the possibility still exists that *P. acnes* may be involved in the pathogenesis of arthritis. Weak arthropathic activity of *P. acnes* peptidoglycan-polysaccharide fragments upon i.p. injection in female Sprague-Dawley rats [45] and tissue destruction in rats after the intraarticular injection of

formalin-killed *P. acnes* cells [46] has been observed.

We have shown that by the alkaline mercuric oxycyanide procedure high molecular weight polysaccharides can be isolated from intact cells as well as culture media. This allows us to dissect from the cellular peptidoglycan antigenic material by peptide bond cleavage, unlike phenol-water extraction. Despite the alkaline conditions, excellent recovery of antigenicity was observed. Even 24 h exposure to the reagent caused no loss in antigenicity, making it a new tool to dissect alkali-stable polysaccharides from their covalent and/or non-covalent association with proteins.

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