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Primary Structure of the Wall Peptidoglycan of Leprosy-Derived Corynebacteria

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The cell walls isolated from axenically grown leprosy-derived corynebacteria were submitted to various chemical and enzymatic degradations. The glycan strands of the wall peptidoglycan are essentially composed of *N*-acetylglycosaminyl-*N*-acetylmuramic acid disaccharide units. Small amounts of *N*-acetylglycosaminyl-*N*-glycolylmuramic acid (less than 10%) were also detected. The muramic acid residues of adjacent glycan strands are substituted by amidated tetrapeptide units which, in turn, are cross-linked through direct linkages extending between the C-terminal D-alanine residue of one tetrapeptide and the *meso*-diaminopimelic acid residue of another tetrapeptide. Such a structure is very similar to that of the wall peptidoglycan found in the taxonomically related microorganisms of the *Corynebacterium*, *Mycobacterium*, and *Nocardia* groups.

The acid-fast gram-positive organism *Mycobacterium leprae* cannot multiply in vitro. It is found in lepers' lesions associated with another type of non-acid-fast, gram-positive microorganism, which can be propagated in axenic cultures (1, 4), cross-react with lepers' and anti-mycobacterial sera (12, 15), are morphologically related to *Corynebacterium diphtheriae* (1, 4), and possess in their cell envelopes components (arabinose, galactose, and mycolic acids) (E. Janczura, C. Abou-Zeid, C. Gailly, and C. Cocito, submitted for publication) that are known to be specific to the *Corynebacterium*, *Mycobacterium*, and *Nocardia* group. Since the wall peptidoglycans of these latter bacteria have been extensively studied (2, 3, 11, 13, 14), experiments were devised to unravel the primary structure of the wall peptidoglycan of several leprosy-derived corynebacteria (LDC) strains. The results of these investigations are presented in this report.

MATERIALS AND METHODS

Bacterial strains. The LDC strains 4, 8, 15, and 19 (previously designated D43, L3, DM-86, and Manga, respectively) were isolated from lepromatous tissues of African patients (J. Delville, University of Louvain) (4). Strain 20 (previously designated Medalle X) was found by C. Reich in a biopsy made in the Philippines (courtesy of L. Barksdale, New York University) (1). These strains were grown in Dubos medium supplemented with horse serum (5%, vol/vol) and sodium succinate (7 mg/ml) in fermentors at 37°C and under forced aeration.

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Isolation and purification of wall peptidoglycan. Exponentially growing bacteria were harvested, suspended in 66 mM sodium phosphate buffer (pH 7.8), and disrupted in a CO_2-cooled vibrating Braun disruptor, type 853032 (five treatments of 2 min each, using 50 g of 0.10-mm glass beads and 10 g, wet weight, of packed cells). After dilution of the homogenates with phosphate buffer, the remaining intact cells and cellular debris were eliminated by filtration of the suspensions through a 2 G Duran sintered glass filter (Jena Glass; Schott, Mainz, Germany), followed by three successive centrifugations at 1,000 × *g* of 5 min each. The crude cell envelopes were then sedimented by centrifugation at 23,000 × *g* for 15 min at 4°C, suspended in phosphate buffer, and incubated sequentially (at 37°C and for 16 h, in each case) with pancreatic deoxyribonuclease I (1 mg/10 g of cell wall material), pancreatic trypsin (150 mg/10 g), and pancreatic chemotrypsin (25 mg/10 g). The three enzymes used in this purification step were from Serva Feinbiochemica, Heidelberg, Germany. The partially purified cell walls were collected by centrifugation at 23,000 × *g* for 15 min at 4°C, repeatedly washed with 1 M NaCl and water, extracted successively with acetone, ether-ethanol (1:1, vol/vol), and chloroform-methanol (2:1, vol/vol), and dried in a stream of air. Further purification was then achieved by mild acid treatment (10 ml of 0.1 N HCl per 100 mg of wall material, at 60°C for 12 h), followed by further delipidation with chloroform-methanol (2:1, vol/vol) for 19 h at 20°C. The walls were collected by centrifugation, washed with acetone, and air-dried.

Amino acid analyses. After hydrolysis with 6 N HCl for 16 h at 105°C, the total amino groups were estimated by the fluorodinitrobenzene technique (8), and the amino acid residues were quantitatively estimated with a 3201 LKB amino acid analyzer (equipped with a column of the cation exchange resin Durrum-LKB and using 66 mM sodium citrate buffers, pH 3.22

and 4.20, at 51 and 72°C, respectively). The same technique permitted quantitative estimation of free NH_3. Characterization of the *meso* and DD isomers of diaminopimelic acid (A_2pm) was performed by descending paper chromatography of acid hydrolysates according to the technique of Rhuland (cited in Janczura et al., submitted for publication).

Sugar analyses. After hydrolysis with 3 N HCl for 3 h at 100°C, the total hexosamines were estimated by the Elson-Morgan procedure (8). Free glucosamine (R_f = 0.2) and muramic acid (R_f = 0.44) were characterized by chromatography of the acid hydrolysates on Whatman no. 1 paper using the solvent 1-butanol-acetic acid-water (3:1:1, vol/vol), followed by staining with paradimethylbenzaldehyde as previously described (J. Falszpan-Wietzerbin, doctoral thesis, University of Paris-South, Orsay, France, 1973). After enzymatic degradation of the glycan moiety of the peptidoglycan, the total reducing groups were estimated with the Park-Johnson procedure (8), and free and peptide-substituted β-1,4-*N*-acetylglycosamyl-*N*-acetylmuramic acid disaccharides were estimated with the Morgan-Elson procedure (8). Free disaccharide units (R_f = 0.32) were also characterized by chromatography on Whatman no. 1 paper, using the same conditions as for glucosamine (see above). In addition, Sharon reagent (2% NaOH in 4:6 propanol-ethanol) (Falszpan-Wietzerbin, thesis) was also used for developing the chromatograms.

Analyses of terminal groups. Terminal NH_2 and COOH groups of peptides were characterized and estimated by dinitrophenylation, followed by acid hydrolysis, and by hydrazinolysis, respectively. For more details, see Ghuysen et al. (8).

Estimation of D-alanine. Free D-alanine was estimated with the D-amino acid oxidase technique (8). The D-lactate dehydrogenase, D-amino acid oxidase, and catalase used were from Boehringer, Mannheim, Germany.

Estimation of glycolyl groups. After hydrolysis of 10 mg of wall with 4 N HCl for 24 h at 100°C, the hydrolysate was filtered on a 0.9-ml column (5 by 0.5 cm) of Dowex 50W-X80, and the column was washed with 1 ml of water. Part of the fraction thus obtained was treated with 2,7-dihydroxynaphthalene in 10 M H_2SO_4, and the spectrophotometric measurements at 530 nm were performed as described in reference 18. Another part of the hydrolysate was submitted to chromatography on Whatman no. 1 paper, using the solvent 1-butanol-acetic acid-water (1:1:1, vol/vol), and detected with bromophenol blue (Falszpan-Wietzerbin, thesis). Glycolic acid had an R_f value of 0.6.

Peptidoglycan hydrolases. The endo-*N*-acetylmuramidases (EC 3.2.1.17) from *Chalaropsis* (9) (crystalline enzyme; a gift from J. A. Hash, Vanderbilt University, Nashville, Tenn.) and *Streptomyces globisporus* (20) (a gift from K. Yokogawa, Dainippon Pharmaceutical Co., Tokyo, Japan) hydrolyze the β-1,4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the glycan strands. The *N*-acetylmuramyl-L-alanine amidase from *Streptomyces albus* G (7) hydrolyzes the amide linkages at the junction between the glycan strands and the peptide units. The DD-carboxypeptidase from *S. albus* G (5) (crystalline enzyme) hydrolyzes the D-Ala-(D)-*meso*-A_2pm interpeptide linkages in the peptide moiety. Amidation of the carbonyl group of *meso*-A_2pm in α position to the peptide bond does not abolish but much decreases the enzyme activity. For more details on the mode of action of these peptidoglycan hydrolases, see reference 6.

RESULTS

Chemical composition of the wall peptidoglycan. On the average (Table 1), the walls isolated from the five strains of LDC examined contained, per mg of dry weight, 0.51 μmol of *meso*-A_2pm, 0.67 μmol of Glu, 1.17 μmol of Ala, and 1 μmol of muramic acid plus glucosamine. LL-A_2pm was not detected by Rhuland's technique (cited in Janczura et al., submitted for publication). On the basis of the action exerted by the DD-carboxypeptidase (see below), A_2pm must occur in the form of the *meso* isomer. The fraction of the wall material, hydrolyzed and purified as described in the legend to Fig. 1, thus appeared to consist of a classical *meso*-A_2pm-containing peptidoglycan of chemotype I (6).

Enzymatic hydrolyses of the wall peptidoglycan. The walls isolated from both LDC strains 8 and 15 (400 mg, dry weight, in 400 μl, final volume, of buffer) were separately treated with the *S. globisporus* *N*-acetylmuramidase (in 20 mM sodium phosphate buffer, pH 6.5, containing 10 μg of enzyme), with the *Chalaropsis* *N*-acetylmuramidase (in 20 mM sodium acetate buffer, pH 5.0, containing 5 μg of enzyme), and with the *S. albus* G DD-carboxypeptidase (in 10 mM Tris-hydrochloride buffer, pH 8.0, containing 5 mM $MgCl_2$ and 30 μg of enzyme). After 20 h at 37°C, the two *N*-acetylmuramidases caused a maximal decrease of the turbidities of the wall suspensions to about 10% of their original values. In parallel to this, reducing groups maximally equivalent to about 0.9 mol of β-1,4-*N*-acetylglycosamyl-*N*-acetylmuramic acid disaccharide units per mol of *meso*-A_2pm were exposed, indicating an almost complete hydrolysis of the sen-

TABLE 1. *Composition of the wall peptidoglycan of five LDC strains*

Strain		Wall peptidoglycan (nmol/mg of purified cell wall)			
No.	Batch	*meso*-A_2pm	Glu	Ala	Total hexosamines
4		550	820	1,500	1,100
8		510	586	1,159	960
15	1	520	520	972	990
	2	544	552	944	1,050
19	1	450	827	1,160	850
	2	504	650	1,150	1,009
20		470	730	1,300	1,010
Average		507	670	1,170	1,000

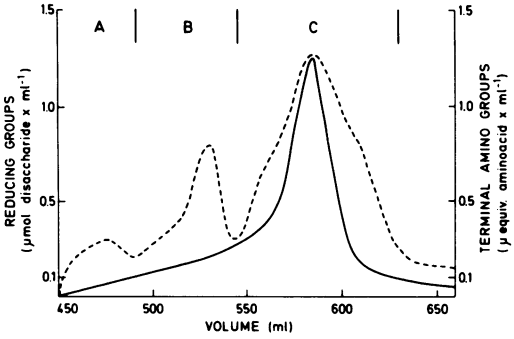

FIG. 1. *Sephadex filtration of the N-acetylmuramidase- and* DD*-carboxypeptidase-treated cell wall of strain 15. Cell walls (100 mg) were suspended in 25 ml of 20 mM sodium acetate buffer (pH 5.0) and incubated with 1.25 mg of Chalaropsis enzyme for 20 h at 37°C. After elimination of the undissolved material (20 mg) by centrifugation, the solution was neutralized with 1 N KOH, supplemented with 10 ml of 10 mM Tris-hydrochloride buffer (pH 8.0) containing 8 mg of the S. albus G enzyme, and incubated for 16 h at 37°C. The resulting solution was filtered in 0.1 M LiCl on a combined G-50 Sephadex column (93 by 2.7 cm)-G-25 Sephadex column (103 by 2.4 cm) system, where the two columns were connected in series in the indicated order (total void volume, 475 ml). Reducing groups (dotted line) and free amino groups (solid line) were estimated in the fractions collected. Fractions A, B, and C were desalted. Fraction C consisted of disaccharide-(amidated) tetrapeptide monomer units.*

sitive glycosidic linkages. Analyses of the terminal amino groups present in the wall digests showed that about 20% of the *meso*-A_2pm residues had one amino group free, thus indicating an average degree of polymerization of 80% cross-linked peptide units for the intact peptide moieties of the wall peptidoglycans. Contrary to the endo-*N*-acetylmuramidases, the *S. albus* G DD-carboxypeptidase caused only a limited extent of clarification of the wall suspensions (20 to 25%). However, after treatment of the isolated walls first with the *Chalaropsis* enzyme (at pH 5.0) and then with the *S. albus* G enzyme (at pH 8.0 and in the presence of 5 mM $MgCl_2$) under the conditions described in the legend of Fig. 1, analyses of the degraded products revealed that the glycan strands had been completely degraded into disaccharide units and that about 50 to 65% of the *meso*-A_2pm residues now occurred at the N-terminal position of the peptide moieties. *S. albus* G DD-carboxypeptidase hydrolyzed the substrate with less efficiency when acting alone than when acting subsequently to muramidase.

Isolation and characterization of the disaccharide-peptide monomer. The isolated walls of LDC strain 10 (100 mg) were submitted to the sequential action of the *Chalaropsis* and *S. albus* G enzymes, and the reaction products were fractionated by Sephadex filtration in 0.1 M LiCl, as described in the legend of Fig. 1. The three fractions, A, B, and C, collected at K_D values of 0.12, 0.22, and 0.66, respectively, were desalted by filtration on Sephadex G-25 in water. Fractions A, B, and C contained 5, 20, and 70%, respectively, of the original wall peptidoglycan. Fraction C was composed of equimolar amounts of glucosamine, muramic acid, L-Ala, Glu, *meso*-A_2pm, D-Ala, and NH_3, and contained 1 equivalent of mono-NH_2-*meso*-A_2pm. It thus consisted of disaccharide-(monoamidated)tetrapeptide units. This disaccharide-peptide monomer was, in turn, submitted to the action of the *N*-acetylmuramyl-L-alanine amidase under the conditions described in the legend of Fig. 2, causing a complete separation between the disaccharide and the peptide units. The degradation products were filtered in water on a column of Sephadex G-25. Fraction D was composed of equimolar amounts of glucosamine and muramic acid and thus consisted of free disaccharide units. These disaccharide units comigrated by paper chromatography with authentic β-1,4-*N*-acetylglycosaminyl-*N*-acetylmuramic acid. Fraction E was composed of equimolar amounts of *meso*-A_2pm, Glu, D-Ala, L-Ala (obtained by subtracting D-Ala from total Ala), and NH_3, and con-

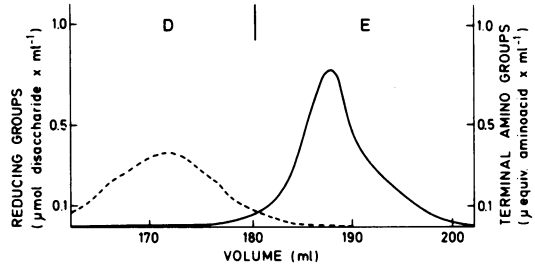

FIG. 2. *Sephadex filtration of the N-acetylmuramyl-*L*-alanine amidase-treated disaccharide-(amidated)tetrapeptide units of the wall peptidoglycan of strain 15. A sample (4.5 μmol) of disaccharide-(amidated)tetrapeptide (fraction C of Fig. 1) was incubated for 6 h at 37°C in 4.5 ml of 50 mM acetate buffer (pH 5.4) containing 750 μl of amidase (total hydrolyzing capacity, 45 μeq of N-acetylmuramyl-*L*-alanine linkages hydrolyzed when acting on the disaccharide peptide monomer β-1,4-N-acetylglycosamyl-N-acetylmuramyl-*L*-Ala-*D*-Glu(NH_2)-*L*-Lys-*D*-Ala). The degradation products were fractionated by filtration, in water, on a Sephadex G-25 column (103 by 2.4 cm; void volume, 165 ml). Reducing groups (dotted line) and free amino groups (solid line) were estimated in the fractions collected. Fraction D consisted of disaccharide units, and fraction E consisted of amidated tetrapeptide units.*

tained 1 equivalent of both N-terminal Ala and mono-NH_2-*meso*-A_2pm per peptide unit. In addition, Ala was the only C-terminal amino acid residue that could be detected by hydrazinolysis. Fraction E thus consisted of (amidated) tetrapeptide units. The tetrapeptide was submitted to paper electrophoresis at pH 6.5 (60 V/cm for 1 h). It behaved as a neutral compound, a property which was compatible with the occurrence of one amide group.

Occurrence of *N*-acetyl- and *N*-glycolylmuramic acid residues. The walls of LDC strain 15 were hydrolyzed with HCl, and the hydrolysate was filtered on Dowex 50W-X80 as described in Materials and Methods. Reaction with dihydroxynaphthalene and spectrophotometric measurements permitted detection of 6 nmol of glycolic acid per mg of walls. Glycolic acid was also detected by paper chromatography, but apparently in much lower amounts. From these experiments it was concluded that a major part of muramic acid occurred as the *N*-acetyl derivative and that about 2 to 10% occurred in the form of the *N*-glycolyl derivative.

DISCUSSION

From the data presented above, it appears that the peptide moiety of the wall peptidoglycan of LDC is of chemotype I (6) with *meso*-A_2pm linkages serving as peptide bridges. The tetrapeptide units also possess one amide substituent, but its location (on the α-carboxyl group of Glu or on that carboxyl group of *meso*-A_2pm which is not involved in the main backbone of the peptide) has not been determined. All the mycobacteria, nocardia, and corynebacteria strains examined so far possess a wall peptidoglycan with the same type of peptide moiety, and the sequence L-Ala-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala (at various levels of amidation) has been assigned to their tetrapeptide units (2, 6, 13, 14, 16, 17, 19).

From the data presented above, it also appears that the glycan moiety of the wall peptidoglycan of LDC contains a small proportion of *N*-glycolylmuramic acid together with a high proportion of the widely occurring *N*-acetylmuramic acid. Previous works have shown that glycolyl groups occur in the wall peptidoglycans of mycobacteria, *Nocardia*, and *Micromonospora*, but not in those of *Streptomyces* or corynebacteria (14; Falszpan-Weitzerbin, thesis). More recently, however, this view had to be revised, as glycolyl groups were detected in small amounts in the cell walls of *Corynebacterium michiganense*, *Corynebacterium sepedonicum*, and *Corynebacterium bovis* and in much larger amounts in those of an unclassified coryneform, IAM 1311 (18). One should note that whereas the genera *Mycobacterium* and *Nocardia* are genetically homogenous (67 to 69 mol% guanine plus cytosine in the DNAs), the genus *Corynebacterium* is highly heterogeneous (52 to 69 mol% guanine plus cytosine) and comprises at least four broad groups related to *C. diphtheriae*, *Corynebacterium renale*, *Corynebacterium genitalium*, and *Rhodococcus phylus* (10, 11). The guanine plus cytosine content of the LDC has been found to be 56 to 60 mol% (P. Danhaive, P. Hoet, and C. Cocito, unpublished data).

Another conclusion of the present work is that the peptidoglycan of LDC and that of *Mycobacterium tuberculosis* are very similar. In this connection, the observed cross-reaction between the walls of LDC and anti-mycobacterial sera (12, 15) is worthy of mention.

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