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Immunochemical Study of the Peptidoglycan of Gram-Negative Bacteria

Hiep NGUYEN-HUY, Charles NAUCIEL, and Camille-Georges WERMUTH

Institut de Bactériologie et d'Immunologie Générale, Faculté de Médecine, Université Louis-Pasteur, Strasbourg

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The specificity of antibodies directed against the peptidoglycan of gram-negative bacteria was studied. The peptidoglycans of *Proteus vulgaris*, *Escherichia coli*, *Moraxella glucidolytica*, *Neisseria perflava*, give identical precipitin reactions. By means of inhibition studies with various peptidoglycan subunits and synthetic peptides, it was shown that the antibodies are essentially directed against the peptide moiety of the peptidoglycan: L-Ala-D-Glu⌊⌋(L)-*meso*A_2pm-(L)-D-Ala, that the peptide reacts better with antibodies when it is not cross-linked, and that the C-terminal portion -*meso*-A_2pm-D-Ala of the peptide is immunodominant. These results explain the immunological identity of the peptidoglycans of gram-negative bacteria, which possess the same peptide subunit. Only weak cross-reactivity was observed with the peptidoglycans of gram-positive bacteria (*Streptococcus faecium*, *Micrococcus lysodeikticus*, *Corynebacterium poinsettiae*) where *meso*-diaminopimelic acid is replaced by L-lysine or L-homoserine. However, the peptidoglycan of *Bacillus megaterium* which possesses the same peptide subunit as gram-negative bacteria, gives only a reaction of partial identity with these bacteria. This result suggests the presence on the peptidoglycan of gram-negative bacteria, of other undefined antigenic determinants.

The peptidoglycan is a component of the bacterial cell wall. It possesses various biological properties [1], particularly adjuvant activity in immune response [2]. The peptidoglycan exhibits a great similarity of structure throughout the bacterial world [3]. It is a macromolecule composed of glycan strands which are interconnected through short peptide chains. The glycan strand is made of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The peptide subunit, linked to the lactyl group of *N*-acetylmuramic acid, is generally a tetrapeptide but in some cases, a tripeptide or a pentapeptide. In gram-negative bacteria, the sequence of the tetrapeptide is L-Ala-D-Glu⌊⌋(L)-*meso*-A_2pm-(L)-D-Ala. Variations in the structure of the peptide can occur among gram-positive species. A great proportion of the peptides are cross-linked. In gram-negative bacteria, there is a direct linkage between *meso*-diaminopimelic acid and D-alanine. In other bacteria, the bridging is mediated by one or several additional amino acids.

Abbreviations. A_2pm, 2,2′-diaminopimelic acid; Boc, *t*-butoxycarbonyl; Hse, homoserine; OBzl, benzyl ester; ONp, *p*-nitrophenyl ester; ONSu, *N*-hydroxysuccinimide ester; Z, benzyloxycarbonyl; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid.

Previous immunochemical studies have dealt with only gram-positive bacteria, most often using group A-variant *Streptococcus* antisera [4–6]. These studies have shown that the majority of the antibodies are directed against a pentapeptide L-Ala-D-Glu⌊⌋L-Lys-D-Ala-D-Ala and that the immunodominant portion was the C-terminal -D-Ala-D-Ala [5]. The present study has been carried out with the peptidoglycan of four species of gram-negative bacteria: *Escherichia coli*, *Proteus vulgaris*, *Moraxella glucidolytica* and *Neisseria perflava*. In the peptidoglycan of these bacteria, no pentapeptide has been found and the amino acid in position 3 is *meso*-diaminopimelic acid. The results indicate that the antibodies elicited by the peptidoglycan of gram-negative bacteria, are directed against the tetrapeptide and that the C-terminal -*meso*-A_2pm-D-Ala plays a major part in the specificity. The cross-reactivity of the antibodies with the peptidoglycans of various structures has been studied.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Proteus vulgaris P 18, *Moraxella glucidolytica*, *Neisseria perflava*, and *Streptococcus faecium* were

isolated in the Institut de Bactériologie (Strasbourg). *Escherichia coli* RE 600 was obtained from the Institut de Biologie Moléculaire (Strasbourg), *Micrococcus lysodeikticus* and *Bacillus megaterium* ATCC 14581 from the Institut Pasteur (Paris) and *Corynebacterium poinsettiae* NCPPB 846 from the Station de Pathologie Végétale de l'INRA (Beaucouzé). The bacteria were grown in a New Brunswick fermentor for 24 h at 37 °C (except for *M. lysodeikticus* and *C. poinsettiae* which were grown at 30 °C). The bacteria were collected with a Sharpless centrifuge and lyophilized.

Preparation of Peptidoglycans

Lyophilized gram-negative bacteria were suspended in distilled water (10 g in 200 ml) and disrupted by ultrasonic treatment for 30 min. Cell walls were isolated by differential centrifugation. The peptidoglycan of *E. coli* and *P. vulgaris* was then purified by treatment with hot sodium dodecylsulphate and trypsin, according to the method of Braun and Rehn [7]. A slight modification of this method [8] was used for *M. glucidolytica* and *N. perflava*. *M. lysodeikticus* and *S. faecium* were treated by 10% trichloroacetic acid (20 min at 100 °C) and trypsin in Tris buffer 0.05 M, pH 8.2 during 2 h at 37 °C (ratio enzyme-substrate 1/25). After addition of 1% sodium dodecylsulphate, the peptidoglycan was washed 5 times in distilled water and lyophilized. In the case of *C. poinsettiae* and *B. megaterium*, the cell walls were isolated (as for gram-negative bacteria) and the peptidoglycan was purified as described by Perkins and Nieto [9].

Preparation of Peptidoglycan Fragments

The peptidoglycan of *E. coli* was digested by egg-white lysozyme (Sigma). The disaccharide-peptide monomer and the bis-disaccharide-peptide dimer were partially separated by gel-filtration on Sephadex G 50 and G 25 [8]. The separation was achieved by paper electrophoresis at pH 1.9 [10]. The fraction containing the disaccharide-peptide monomer was submitted to paper chromatography [11] in order to separate the disaccharide-tetrapeptide GlcNAc-β-1,4-MurNAc-L-Ala-D-Glu┌(L)-*meso*-A_2pm-(L)-D-Ala from the the disaccharide-tripeptide GlcNAc-β-1,4-MurNAc-L-Ala-D-Glu┘-(L)-*meso*-A_2pm.

The disaccharide-tripeptide and the disaccharide-tetrapeptide were treated with NH_3 which causes cleavage of lactyl-peptides from the disaccharide [12]. After elimination of NH_3 by lyophilization, the lactyl-tripeptide and the lactyl-tetrapeptide were isolated by thin-layer chromatography on Silicagel 60 (Merck) in the following solvent system: isobutyric acid/1 N NH_3 (5/3, v/v).

The amino acid and amino sugar content of these fragments has been controlled by chemical analysis.

Amino Acids and Amino Acid Derivatives

L-Alanine, D-alanine, *meso*-diaminopimelic acid, D-glutamic acid and N^{α}-Boc-N^{ε}-Z-L-Lys-ONSu were purchased from Fluka, D-Ala-D-Ala from Fox Chemical Company and benzyloxycarbonyl-L-alanine from Mann Research Laboratories.

Z-L-Ala-ONp was obtained by coupling benzyloxycarbonyl-L-alanine with *p*-nitrophenol [13].

Boc-D-Glu-OBzl and *p*-tosylate, D-Ala-OBzl were prepared according to methods described for L isomers [14, 15].

Synthesis of L-Ala-D-Glu and L-Ala-D-Glu┘-L-Lys-D-Ala

Active ester methods were used for coupling of amino acids [13, 16]. *t*-Butoxycarbonyl group was eliminated by HCl in glacial acetic acid. Removal of benzyloxycarbonyl and benzyl groups was achieved by catalytic hydrogenation and checked by nuclear magnetic resonance.

Z-L-Ala-D-Glu-OBzl (I) was obtained by reacting Z-L-Ala-ONp with D-Glu-OBzl. This product has the following properties: m.p. 138 °C; $[\alpha]_D^{22} = +3.3°$ ($c = 2$; ethyl acetate). Analysis: calculated for $C_{23}H_{26}N_2O_7$: C 62.43, H 5.92, N 6.33; found: C 62.37, H 6.06, N 6.50.

Removal of protecting groups yielded the dipeptide L-Ala-D-Glu which was purified by paper chromatography in *n*-butanol/acetic acid/water (3/1/1; v/v/v).

L-Lys(Z)-D-Ala-OBzl (II) was obtained by reacting N^{α}-Boc-N^{ε}-Z-L-Lys-ONSu with D-Ala-OBzl and then removing the *t*-butoxycarbonyl group. This compound has the following properties: m.p. 81.3 °C; $[\alpha]_D^{22} = +6.7°$ ($c = 2$; ethyl acetate). Analysis: calculated for $C_{24}H_{31}N_3O_5$: C 65.28, H 7.08, N 9.52; found: C 65.45, H 7.15, N 9.70.

Z-L-Ala-D-Glu-OBzl┘-L-Lys(Z)-D-Ala-OBzl was obtained by coupling compounds I and II by the dicyclohexylcarbodiimide method [17]: m.p. 184 °C; $[\alpha]_D^{22} = +5.3°$ ($c = 2.5$; dimethylformamide). Analysis: calculated for $C_{47}H_{55}N_5O_{11}$: C 65.18, H 6.40, N 8.09; found: C 65.14, H 6.49, N 8.07.

Protecting groups were removed and L-Ala-D-Glu┘-L-Lys-D-Ala was purified on a Dowex 50X4 column [5].

Analytical Methods

Quantitative determination of amino acids was performed after 18 h hydrolysis at 105 °C in 6 N HCl, either with a Beckman Unichrom amino-acid analyzer [8] or by thin-layer chromatography, after coupling with fluorodinitrobenzene [18]. Amino sugars were determined by the Morgan-Elson reaction [18].

Immunological Methods

Rabbits were immunized with sodium-dodecylsulphate-treated cell walls of gram-negative bacteria (*P. vulgaris*, *M. glucidolytica*, *N. perflava*). Intravenous injections of 1 mg were given 3 times a week during several weeks. Unless otherwise indicated the immunological studies were made with the same anti-(*P. vulgaris*) serum. Before reaction with antisera, purified peptidoglycans were suspended in saline and treated by an ultrasonic disintegrator. Antibodies were detected by the ring test. Immunodiffusion was carried out by the Ouchterlony's method, in 1% agarose. Quantitative precipitin tests were performed as follows. Increasing amounts of purified peptidoglycan were added to 0.1 ml of antiserum and the volume was adjusted to 0.4 ml with saline. After incubation at 37 °C for 1 h and 4 °C for 48 h, precipitates were collected by centrifugation, washed 3 times with saline, and dissociated in 0.5 ml 0.1 N NaOH. Insoluble peptidoglycan was eliminated by centrifugation and the antibody amount was determined by measuring the absorbance at 280 nm and subtracting the absorbance at 320 nm [19].

Quantitative precipitin inhibition tests were performed as follows. Increasing amounts of inhibitors dissolved in saline were added to a series of tubes containing 0.1 ml of antiserum. After 1 h incubation at 37 °C, purified peptidoglycan was added (in an amount corresponding to the antigen-antibody equivalence). The volume was adjusted to 0.4 ml with saline and the tubes were processed as for quantitative precipitation. Inhibition was determined by comparison to the amount of antibody recovered without inhibitor.

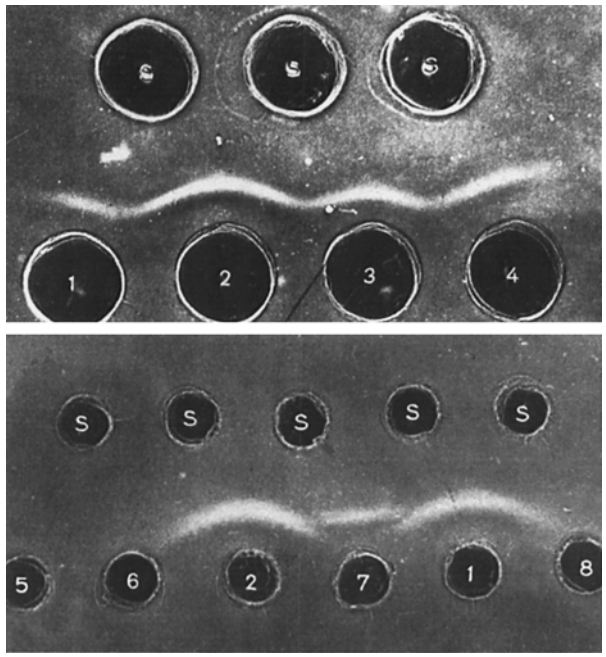

Fig. 1. *Immunodiffusion reaction between anti-*(P. vulgaris) *serum (S) and various peptidoglycans.* (A) *N. perflava* (1), *P. vulgaris* (2), *M. glucidolytica* (3) and *E. coli* (4). (B) *S. faecium* (5), *M. lysodeikticus* (6), *P. vulgaris* (2), *B. megaterium* (7), *N. perflava* (1) and *C. poinsettiae* (8)

RESULTS

Preliminary experiments have shown that purified peptidoglycan of gram-negative bacteria were poorly immunogenic. Good antisera were obtained by immunization with partially purified preparations (sodium-dodecylsulphate-treated cell walls). These antisera have been reacted with purified peptidoglycans of homologous or heterologous bacteria in order to minimize the interference of antibodies directed against contaminants of the preparations used for immunization.

Comparative Study of Peptidoglycans of Gram-Negative Bacteria

The peptidoglycan of *P. vulgaris*, *E. coli*, *M. glucidolytica* and *N. perflava* were used. The results of chemical analysis were similar for these bacteria. The amount of glutamic acid was around 1000 nmol/mg and the molar ratio of glutamic acid, *meso*-diaminopimelic acid and alanine was 1/1/1.70. Among the amino acids which are not constituents of the peptidoglycan, a small amount of L-lysine (0.04 to 0.12 mol per mol of glutamic acid) was found and only traces of other amino acids. This indicates the absence of meaningful proteic contamination.

The immunodiffusion reaction between these peptidoglycans and a serum directed against the peptidoglycan of *P. vulgaris* is shown in Fig. 1 A. Only one precipitin line is apparent for each antigen and all the lines fuse. Similar results have been obtained with sera directed against the peptidoglycan of *M. glucidolytica* and of *N. perflava*.

The results of quantitative precipitin tests are reported in Fig. 2. From the absorption coefficient of rabbit immunoglobulins [20] the amount of precipitable antibodies in the sera was found to be 2.8 mg/ml. The peptidoglycans of the four gram-negative bacteria precipitate almost the same amount of antibodies. This indicates that these peptidoglycans bear the same antigenic determinants. However, the slope of the precipitin curve are different and this point will be discussed further.

Evidence that the Peptide Subunit is the Major Antigenic Determinant

The antisera against the peptidoglycan of *P. vulgaris* was incubated with various amounts of peptido-

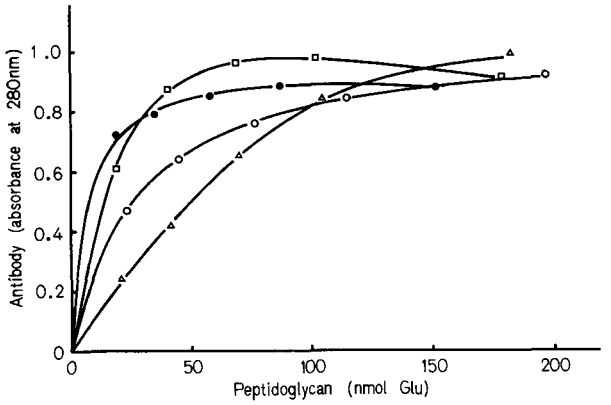

Fig. 2. *Quantitative precipitin reaction between anti-*(P. vulgaris) *serum and the peptidoglycan of* E. coli (●), P. vulgaris (□), M. glucidolytica (○), N. perflava (△)

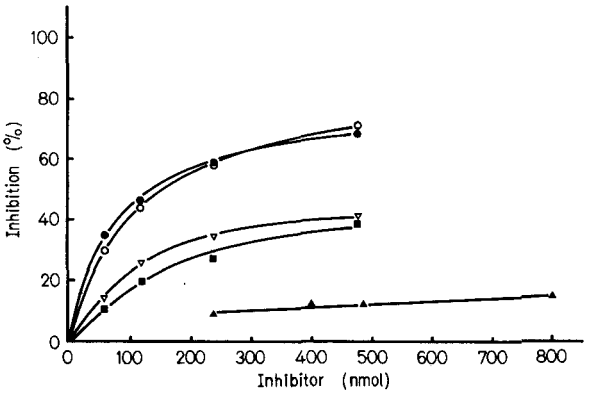

Fig. 3. *Inhibition of the precipitin reaction between anti-*(P. vulgaris) *serum and the peptidoglycan of E.* coli. Inhibitors: disaccharide-tetrapeptide (○), bis-disaccharide-tetrapeptide dimer (▽), lactyl-tetrapeptide (●), lactyl-tripeptide (■), synthetic peptide L-Ala-D-Glu ┌L-Lys-D-Ala (▲)

glycan fragments from *E. coli* and precipitation was performed with the peptidoglycan of *E. coli*. The results of these experiments are reported in Fig. 3. Inhibition with 480 nmol of the disaccharide-tetrapeptide monomer reaches 70%. The lactyl-tetrapeptide gives the same inhibition curve. This indicates that the antibodies are directed only against the peptidic moiety of the peptidoglycan. However, the bis-disaccharide-tetrapeptide dimer (which contains two tetrapeptides by mole) is much less inhibitory than the disaccharide-tetrapeptide monomer. This means that most of the antibodies combine better with noncross-linked tetrapeptides than with cross-linked tetrapeptides. The major antigenic determinant in this system appears to be the noncross-linked tetrapeptide L-Ala-D-Glu ┌(L)-*meso*-A_2pm-(L)-D-Ala. It is known that the degree of cross-linkage between peptides varies among bacterial species [21]. This can be estimated from the proportion of monomeric subunits obtained by lysozyme digestion. In the case of *E. coli*, *P. vulgaris*, *M. glucidolytica* and *N. perflava*, the proportions are respectively: 40%, 32%, 31% and 21% [8, 22]. There is a good correlation between these findings and the slopes of the precipitin curves in Fig. 2. The greatest slopes are obtained with the peptidoglycans possessing the highest proportion of monomeric subunits.

Role of Various Amino Acids in Antigen Specificity

The lactyl-tripeptide is a less efficient inhibitor of precipitation than the lactyl-tetrapeptide (Fig. 3). This result shows the contribution of the C-terminal D-alanine to antigenic specificity. The synthetic peptide L-Ala-D-Glu ┌L-Lys-D-Ala is a very poor inhibitor (Fig. 3). This indicates the major contribution of *meso*-diaminopimelic acid. However, inhibition with 2000 nmol of D-alanine or *meso*-diaminopimelic acid separately is less than 10%. The synthetic dipeptides L-Ala-D-Glu and D-Ala-D-Ala are also poor inhibitors (less than 5% with 800 nmol).

Study of the Cross-Reactivity with the Peptidoglycan of Various Gram-Positive Bacteria

The peptidoglycan of the following bacteria were used: *B. megaterium*, *S. faecium*, *M. lysodeikticus*, *C. poinsettiae*. Except for *B. megaterium*, these bacteria possess a peptide structure which is different from the peptide of the gram-negative bacteria previously studied. The structure of these peptides is represented in Table 1. The peptides of *S. faecium*, *M. lysodeikticus* and *C. poinsettiae* are cross-linked through additional amino acids [3]. However, these bridges do not play a part in the immunological reaction, because the antibodies which are used were raised against a peptidoglycan *(P. vulgaris)* which does not contain these additional amino-acids. The results of the analysis of the peptidoglycans of gram-positive bacteria have shown that the amount of glutamic acid was about 900 nmol/mg and that the molar ratio of the constituents were in good accordance with the values reported in the literature.

Immunodiffusion (Fig. 1 B) and quantitative precipitin tests (Fig. 4) show that the peptidoglycan of *B. megaterium* reacts strongly with the serum directed against the peptidoglycan of *P. vulgaris*. However, only a part of the antibodies are precipitated by *B. megaterium* and a spur can be seen in immunodiffusion reaction. The peptidoglycans from the other bacteria (which do not contain *meso*-diaminopimelic acid) give only a faint cross-reactivity in quantitative pre-

Table 1. *Structure of the peptide subunit of the peptidoglycans*

Bacteria	Peptide subunit
Gram-negative bacteria	L-Ala-D-Glu ┌(L)-*meso*-A_2pm-(L)-D-Ala
Bacillus megaterium	L-Ala-D-Glu ┌(L)-*meso*-A_2pm-(L)-D-Ala
Streptococcus faecium	L-Ala-D-Glu-NH_2 ┌L-Lys-D-Ala
Micrococcus lysodeikticus	L-Ala-D-Glu-Gly ┌L-Lys-D-Ala
Corynebacterium poinsettiae	Gly-D-Glu ┌L-Hse-D-Ala

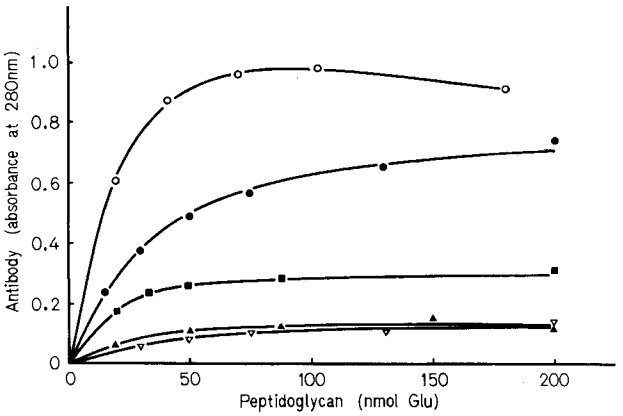

Fig. 4. *Quantitative precipitin reaction between anti-*(P. vulgaris) *serum and the peptidoglycan of P.* vulgaris (○), B. megaterium (●), S. faecium (■), M. lysodeikticus (▲), C. poinsettiae (▽)

cipitation. No precipitin line appears in immunodiffusion reaction.

DISCUSSION

The antibodies elicited by the peptidoglycan of *Proteus vulgaris* appear to be directed essentially against the peptide moiety. The C-terminal D-alanine takes part in the antigenic specificity since the lactyltripeptide is less inhibitory that the lactyl-tetrapeptide. *meso*-Diaminopimelic acid is of major importance, since a synthetic tetrapeptide containing L-lysine instead of *meso*-diaminopimelic acid (found in the natural peptide) is a very weak inhibitor. This point is corroborated by the finding that the antiserum reacts only to a small extent with peptidoglycans which do not contain *meso*-diaminopimelic acid. Thus, the serum is able to discriminate (especially in immunodiffusion reaction) peptidoglycans possessing *meso*-diaminopimelic acid from others.

The contribution to immunological specificity of the two amino acids which are on the C-terminal end has been demonstrated. The role of the N-terminal portion (L-Ala-D-Glu) has not been determined precisely. However, the weak inhibitory effect of a synthetic dipeptide with the same structure indicates that its role is probably not of major importance. Thus the size of the antigenic determinant is a sequence comprised between 2 and 4 amino acids.

The present studies have shown that the peptidoglycans of *E. coli*, *P. vulgaris*, *M. glucidolytica*, and *N. perflava* are antigenically identical. This is in good accordance with chemical studies demonstrating that these bacteria possess the same peptide subunit [8, 23]. An unexpected finding is that *B. megaterium* which also contains the same peptide subunit [10] displays a pattern of partial identity in immunodiffusion reaction and in quantitative precipitation. The antigenic determinant which is present on the peptidoglycan of the gram-negative bacteria and missing in *B. megaterium* is unknown. It has been shown that in *E. coli* and in several other gram-negative bacteria, a lipoprotein is covalently linked to the peptidoglycan [7, 24, 25]. This lipoprotein is attached through its C-terminal L-lysine to the *meso*-diaminopimelic acid of about every tenth peptide subunit. The trypsin digestion performed during the purification of peptidoglycan leaves the L-lysine residue attached to the peptidoglycan [7]. It is worthy of note that a small amount of L-lysine has been detected in the purified peptidoglycans of the gram-negative bacteria that we have used. Thus it is possible that the presence of the residual L-lysine on some peptide subunits gives rise to an antigenic determinant which is absent in *B. megaterium*.

The antigenic specificity of the peptidoglycan of gram-negative bacteria appears to be different from that which has been reported in other studies with gram-positive bacteria. These studies have been conducted mostly with sera obtained after immunization with group A-variant *Streptococcus*. The antibodies were also mainly directed against the peptide moiety but the immunodominant portion was the -D-Ala-D-Ala C-terminal sequence of the pentapeptide L-Ala-D-Glu ┌L-Lys-D-Ala-D-Ala [5]. In our system, the dipeptide D-Ala-D-Ala was devoid of inhibitory activity. However there are some similarities in the results obtained with gram-negative and gram-positive bacteria. In both cases, the immunodominant part of the antigenic determinant is the C-terminal end of the peptide. The inhibition experiments with the disaccharide-peptide monomer and with the bis-disaccharide-peptide dimer have shown that in gram-negative bacteria the antibodies are directed essentially against the noncross-linked peptide. With group A-variant *Streptococcus* the antibodies are directed against the pentapeptide which is a less common subunit than the tetrapeptide L-Ala-D-Glu ┌L-Lys-D-Ala [5]. It is known that in group A-variant *Streptococcus* and some other gram-positive bacteria the non cross-linked peptides are pentapeptides. The cross-

linkage between peptide subunits is achieved by a transpeptidase. During this reaction, the fifth amino-acid of the pentapeptide is removed [21]. Thus in gram-positive bacteria as well, antibodies are directed against the non cross-linked peptide.

The tridimensional structure of the peptidoglycan is not known. However in a space-filling molecular model which has been proposed [26] the peptide has the shape of a T. The stem of the T linked to the glycan strand is made of L-Ala-D-Glu and the crossbar contains the amino acids of the C-terminal end. This C-terminal portions appears to be more accessible. This is in good accordance with the fact that this portion is immunodominant. When the peptides are cross-linked access to this portion is blocked.

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H. Nguyen-Huy and C. Nauciel, Institut de Bactériologie, Faculté de Médicine, Université Louis-Pasteur, 3 Rue Koeberlé, F-67000 Strasbourg, France

C.-G. Wermuth, Laboratoire de Chimie Organique, Faculté de Pharmacie, Université Louis-Pasteur, 3 Rue de l'Argonne, F-67083 Strasbourg-Cedex, France