Digestion of *Streptococcus pneumoniae* Cell Walls with Its Major Peptidoglycan Hydrolase Releases Branched Stem Peptides Carrying Proinflammatory Activity*†*

(Received for publication, December 1, 1998, and in revised form, February 12, 1999)

Paul Anthony Majcherczyk‡, Hanno Langen§, Didier Heumann‡, Michael Fountoulakis§, Michel Pierre Glauser†, and Philippe Moreillon‡

From the ‡Division of Infectious Diseases, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland and §Pharmaceutical Research-Gene Technologies, F. Hoffman-La Roche Ltd., CH-4070 Basel, Switzerland

The peptidoglycan of Gram-positive bacteria is known to trigger cytokine release from peripheral blood mononuclear cells (PBMCs). However, it requires 100–1000 times more Gram-positive peptidoglycan than Gram-negative lipopolysaccharide to release the same amounts of cytokines from target cells. Thus, either peptidoglycan is poorly active or only part of it is required for PMBC activation. To test this hypothesis, purified *Streptococcus pneumoniae* walls were digested with their major autolysin N-acetylmuramoyl-L-alanine amidase, and/or muramidase. Solubilized walls were separated by reverse phase high pressure chromatography. Individual fractions were tested for their PMBC-stimulating activity, and their composition was determined. Soluble components had a $M_r$ between 600 and 1500. These primarily comprised stem peptides cross-linked to various extents. Simple stem peptides ($M_r < 750$) were 10-fold less active than undigested peptidoglycan. In contrast, tripeptides ($M_r > 1000$) were $\approx 100$-fold more potent than the native material. One dipeptide (inactive) and two tripeptides (active) were confirmed by post-source decay analysis. Complex branched peptides represented $\leq 2\%$ of the total material, but their activity (w/w) was almost equal to that of LPS. This is the first observation suggesting that peptidoglycan stem peptides carry high tumor necrosis factor-stimulating activity. These types of structures are conserved among Gram-positive bacteria and will provide new material to help elucidate the mechanism of peptidoglycan-induced inflammation.

Inflammation is a complex, nonspecific host defense response to pathological situations such as infection. When localized, inflammation is beneficial because it helps eradicate the invading organisms at the infected site (1). In severe situations, however, the deregulation of numerous host defense factors and soluble cellular mediators, such as cytokines, may result in a dramatic cardiovascular collapse called septic shock (2, 3). The cytokine tumor necrosis factor (TNF)† was shown to play a critical role in triggering such an event (4, 5). Septic shock is a classical response to severe infections due to Gram-negative bacteria, in which the outer membrane lipopolysaccharide (LPS) is the principal bacterial component triggering cytokine release from peripheral blood mononuclear cells (PBMCs) and other responsive cells (6–9). To stimulate cytokine release, LPS binds to the cell surface protein CD14 (10), a glycosylphosphatidylinositol-linked determinant that presumably requires additional transmembrane elements such as the recently described Tol receptor (11) to trigger cell response. *In vitro* LPS also binds to lipopolysaccharide-binding protein, an acute phase protein that catalyzes the binding of LPS to CD14 (12).

Septic shock is also a feature of severe Gram-positive infections (13–16). In recent years, Gram-positive pathogens have become more frequent than Gram-negative bacteria as a cause of nosocomial infections and shock (17, 18). This is particularly true in intensive care unit patients and in neutropenic patients, two subgroups with impaired host defenses that are especially prone to develop severe sepsis. However, in contrast to their Gram-negative counterparts, Gram-positive bacteria do not contain LPS. In certain cases, highly pathogenic agents such as *Staphylococcus aureus* and *Streptococcus pyogenes* produce toxins that may act as superantigens and trigger an overwhelming nonspecific T-cell-mediated inflammatory response (7, 13). With less pathogenic agents, such as viridans group streptococci, triggering of inflammation and shock might be due to other cellular components, including the thick Gram-positive peptidoglycan. Gram-positive cell walls were shown to stimulate TNF release and inflammation in several *in vitro* and *in vivo* systems, including experimental meningitis, experimental arthritis, and experimental septic shock (19–21).

To study the mechanism of peptidoglycan-induced inflammation, different types of Gram-positive wall materials were utilized. On the one hand, large molecules such as insoluble cell walls and soluble peptidoglycan released from penicillin-treated staphylococci allowed the demonstration that CD14 was also a receptor for Gram-positive wall products (22, 23). On the other hand, the small molecule muramoyl dipeptide was shown to be the minimal common peptidoglycan structure carrying immunomodulatory activity (24). However, while very useful, these compounds might not be ideal for solving the structure-activity relationship between the wall degradation products occurring in the nature and their cytokine-releasing capacity. First, molecules such as soluble peptidoglycan are very large ($M_r \geq 125,000$) (25, 26) and might be too complicated for refined analysis. Second, muramoyl dipeptide is not a nat-

---

*This work was supported by Swiss National Fund for Scientific Research Grants 3200-040836.94 and 3200-052561.97. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Division of Infectious Diseases, CHUV, CH-1011 Lausanne, Switzerland. Tel.: 41 21 314 3020; Fax: 41 21 314 1036; E-mail: Philippe.Moreillon@chuv.hospvd.ch.

†‡ The abbreviations used are: TNF, tumor necrosis factor; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid.
Inflammatory Subcomponents from S. pneumoniae Peptidoglycan

To further explore this question, we attempted to isolate and characterize small proinflammatory peptidoglycan fragments resulting from the digestion of pneumococcal walls with their native autolysins, thereby liberating products of digestion that are likely to be found in vivo. Specifically, *Streptococcus pneumoniae* contains a major autolytic enzyme (an N-acetylmuramoyl-l-alanine amidase, which hydrolyzes the bonds between the glycan chain and the stem peptides) that is responsible both for wall solubilization and cell lysis during the stationary growth phase or during β-lactam treatment (28–30).

The role of this autolytic enzyme in pneumococcal pathogenesis has been demonstrated (31). In certain experiments, a second enzyme (M1-muramidase) that hydrolyzes the glycan chain was used in parallel. M1-muramidase is not formally a pneumococcal enzyme, but it has a functional equivalent in these organisms (32). The resulting soluble fragments were separated by reverse phase high pressure chromatography (HPLC). Individual fractions were tested for their TNF-releasing activity, and the molecular structure of single components carrying inflammatory power was analyzed by mass spectrometry (MS), amino acid and amino sugar analysis, and postsource decay analysis.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Growth Conditions—The capsulated lipoprotein positive *S. pneumoniae* strain R6 was used (33). Bacteria were grown in the chemically defined liquid medium Cden (44). Growth of the cultures was followed by their optical density at 620 nm (*A* 620) with a spectrophotometer (Sequioa-Turner, Moutainville, CA). The cells were stored at −70 °C in Cden containing 20% glycerol.

In certain experiments a label was introduced into the cell walls by the following methods. (i) To label the glycan chains, N-acetyl-D-[1-3H]glucosamine (Amersham Pharmacia Biotech) was added to the culture to a final concentration of 250 μCi/μl and 1.52 μl/μl. (ii) To label the stem peptides, t-1,4-5-3Hlysine (Amersham Pharmacia Biotech) was added to a final concentration of 250 μCi/μl and 0.45 μl/μl. The cultures were allowed to grow for 4–5 generation times in the presence of the label before being harvested as described below.

Preparation and Purification of Cell Walls—Cell walls were prepared as described previously (34). Briefly, organisms in the early logarithmic phase (A 620 of 0.5) were rapidly cooled in an ice/ethanol bath and harvested by centrifugation for 10 min at 16,000 × g and 4 °C. The cells were resuspended in endotoxin-free water, and intrinsic autolytic enzymes were inactivated by pouring the suspension down a tube into boiling SDS (4%, final concentration). After 15 min of boiling, the SDS was removed by extensive washing with water, and the cells were broken open by vortexing with an equal volume of acid-washed glass beads. Typically, 600 μl of wall digest, into a C18 reverse phase column (SuperPac Sephasil C18, 5 μm, 4 × 250-mm column, Amersham Pharmacia Biotech) protected with a guard cartridge (C18, 5 μm, 4 × 10 mm). The mixture was separated using a linear gradient of 0–15% acetonitrile in 0.1% trifluoroacetic acid over 100 min with a flow rate of 0.5 ml/min. Detection was at 210 nm. 1-min fractions were collected. The solvent was removed by rotary evaporation. The amount of material present in each fraction was too small to be measured either by weight or by radioactive counts and was therefore calculated by the percentage area method.

**Preparation and Purification of Wall Digestes by HPLC and Identification of Specific Fractions**—The HPLC system (Hitachi Instruments, Ichige, Hitachinaka, Japan) consisted of the L-7200 autosampler, the L-7100 gradient pump, with low pressure mixing, and the L-7400 UV detector. Column temperature was maintained at 25 °C using a cooler (LabSource, Reinach, Switzerland). The results were analyzed using the D-7000 HPLC System Manager program (Hitachi).

Separation was performed by injection of a 100-μl sample, containing 100 μg of wall digest, into a C18 reverse phase column (SuperPac Sephasil C18, 5 μm, 4 × 250-mm column, Amersham Pharmacia Biotech) protected with a guard cartridge (C18, 5 μm, 4 × 10 mm). The mixture was separated using a linear gradient of 0–15% acetonitrile in 0.1% trifluoroacetic acid over 100 min with a flow rate of 0.5 ml/min. Detection was at 210 nm. 1-min fractions were collected. The solvent was removed by rotary evaporation. The amount of material present in each fraction was too small to be measured either by weight or by radioactive counts and was therefore calculated by the percentage area method. Knowing the weight of material loaded, the amount of material present in each fraction was deduced. The samples were dissolved in 100 μl of water and stored at −20 °C.

For the isolation of specific fractions, the HPLC system was analyzed by the following means. First their content in amino acids was determined using the OPAC postcolumn derivatization method following hydrolysis of the samples for 24 h in 6 N HCl at 110 °C (37). Their molecular mass was measured by matrix-assisted laser desorption ionization time of flight MS, using a Voyager-Elite TOF mass spectrometer (PerSpective Biosystems Inc., Farmingham, MA) operating in the positive ion mode and using 3-nitrobenzyl alcohol acid matrix. The power of the laser was set to 1,600 units, and the accelerating voltage was set to 20,000 V. Postsource decay analysis was performed on certain fractions. N-Acetyl-D-glucosaminyl-(61–4)N-acetylmuramoyl-l-alanyl-d-isoglutaminyl (Calbiochem) was used as a standard (M, 694.7).

**Preparation, Stimulation of Human PMCs or Whole Blood from Rabbits, and Measurement of TNF-α Release—Human PMCs were extracted from heparinized blood of healthy volunteers by Ficoll-Hypaque (Seromed, Munich, Germany) density gradient centrifugation as described by Heumann et al. (22). The cells were suspended in RPMI 1640 medium (Life Technologies, Inc.) and distributed into the wells of a flat bottomed 96-well tissue culture plate at a concentration of 0.5 × 10⁵ cells/well. Each well contained a final volume of 200 μl, which comprised the RPMI medium (140 μl), plasma from the donor (20 μl), and sample (20 μl). LPS from *E. coli* O111 (Sigma) in the concentration range 0.01–100 ng/ml was used as a positive control. PMCs incubated with plasma or with medium alone were used as negative controls. The plates were incubated at 37 °C in an atmosphere of 5% CO₂. After 8 h of incubation, samples (20 μl) of the supernatants were taken, diluted 20-fold in RPMI medium, and stored at −80 °C for measurement of TNF-α concentrations.

For the whole blood assay, heparinized rabbit blood was diluted 1-fold in RPMI 1640 medium supplemented with 10% fetal calf serum as described (38). The diluted blood was distributed into tissue culture plates (200 μl/well). Samples (20 μl) were added, and the plates were incubated as above for 6 h. The plates were centrifuged for 5 min, and samples of the supernatant were diluted and stored as described above.
The chromatography conditions used in these experiments. The generated by this treatment could not be properly resolved by residual peptides (34). However, although amidase solubilized and glycan chains decorated or not with teichoic acids and/or standard. The activity on murine fibroblasts. Recombinant human TNF was used as a

Solubilization and HPLC Separation of Cell Walls—Fig. 2 depicts the solubilization steps used in these experiments. Purified insoluble pneumococcal walls (step 1 in Fig. 2) digested with amidase (step 2 in Fig. 2) produced a wealth of soluble fragments comprising free stem peptides, separated from the glycan backbone at the N-acetylmuramoyl-L-alanine bonds, and glycan chains decorated or not with teichoic acids and/or residual peptides (34). However, although amidase solubilized >90% of the walls (step 2 in Fig. 2), the soluble fragments generated by this treatment could not be properly resolved by the chromatography conditions used in these experiments. The profile of this separation is shown in Fig. 3a.

We reasoned that this might be due to some interference of the undigested glycan backbone (step 2 in Fig. 2) with the C18 column. To circumvent this problem, two strategies were adopted. In the first, the amidase-solubilized walls were submitted to a second digestion with muramidase (step 3A in Fig. 2), an enzyme that hydrolyzes the 1–4 β-bonds between the N-acetylmuramoyl and N-acetylg glucosamine residues of the glycan chain. The profile of this second separation is depicted in Fig. 3b. In contrast to Fig. 3a, Fig. 3b indicates that doubledigested wall material could now be resolved by HPLC. The chromatogram was complex, indicating that the peptidoglycan contained a variety of subcomponents. Nevertheless, this profile was perfectly reproducible in terms of both intrerrcell wall batch resolution and over at least four independent experiments.

A second strategy (step 3B in Fig. 2) to encompass the interference of the undigested glycan chains consisted of removing the glycans from the amidase digest by serial precipitation in water/acetonitrile/propan-2-ol (as described under “Experimental Procedures”) and then analyzing only the peptide-enriched fraction by HPLC. Fig. 3c depicts the result of this separation. It can be seen that this chromatogram resembled very much that of Fig. 3b, indicating that the stem peptides of the cell walls were the prevalent species resolved with both of these methods.

TNF-releasing Activity of Individual HPLC Fractions—In a first series of experiments, we determined the TNF-releasing activity of 1-min fractions collected from the chromatogram presented in Fig. 3b. Individual fractions were exposed to human PBMCs as described. Fig. 4 indicates that the TNF-releasing activity was very low for material eluting before 30 min, whereas it rose considerably later on, i.e. up to 100-fold above background levels. Since the chromatograms resulting from the separation of either double-digested or single amidase-digested peptidoglycan were almost superimposable, we repeated the stimulation experiment with 1-min fractions of both of these enzymatic digests. In this second series of experiments, a new column was used, which resulted in a slight increase in retention times. Fig. 5 presents these results. In these experiments, the TNF assay was performed with whole blood from rabbits, a system that gave similar results as human PBMCs but was much more convenient for screening large numbers of fractions. It can be seen that not only the chromatograms of double-digested or single amidase-digested peptidoglycan were very

### Table I

| Stimulant                        | MSCP<sub>50</sub><sup>a</sup> (ng/ml) |
|---------------------------------|--------------------------------------|
| E. coli O111 LPS                | 0.1                                  |
| Pneumococcal purified walls     | 100                                  |
| Insoluble cell walls            | 100                                  |
| Amidase-solubilized walls       | 100                                  |
| Pneumococcal HPLC separated walls† | ≥100                               |
| HPLC peaks 1–8                  | ≤10                                  |
| Individual peaks<sup>c</sup>    |                                      |
| Peak 5                          | >100                                 |
| Peak 13                         | <10                                  |
| Peak 20                         | <1                                  |

<sup>a</sup> The relative stimulatory potency was expressed as the minimum concentration of material required to induce at least a 10-fold increase in TNF levels above background (minimum stimulatory concentration 10×, or MSC<sub>50</sub>). Values are means of at least three experiments.

<sup>b</sup> Peak numbers refer to the HPLC profiles depicted in Figs. 3 and 4.

<sup>c</sup> Peaks containing a single component as assessed by MS (see Table II).
similar, but their profiles of TNF-release were comparable as well. This was consistent with the fact that the peptide-enriched material contained active subcomponents similar to those observed in the double-digested material.

**Quantitation of TNF-releasing Activity in Separate HPLC Fractions**—To more precisely evaluate the specific TNF-releasing activity of the material present in individual HPLC fractions (Fig. 4), the content of these fractions was quantified as follows. First, the relative contribution of each peak to the whole chromatogram was determined by comparing their areas under the curve to that of the whole HPLC profile using the D-7000 HPLC System Manager software (Hitachi). This permitted the calculation of the percentage of material present in each fraction as compared with the total material loaded on the column. Using this information, the concentration of material present in each fraction was then calculated, and the specific TNF-releasing activity of the fractions was determined.

Table I presents these results. It indicates an appraisal of the relative stimulatory potency of the various materials tested in the present experiments by denoting the minimum concentra-
Inflammatory Subcomponents from S. pneumoniae Peptidoglycan

Fig. 5. TNF released from rabbit blood following stimulation by 1-min fractions collected from the separation of either double-digested wall or single amidase-digested wall enriched for peptides. The wall components were separated on a new column, which resulted in a marginal increase in retention times compared with Fig. 4. Fractions were analyzed by MS and tested for stimulation of TNF release from rabbit blood. Similar profiles were obtained irrespective of whether double-digested walls (a) or single amidase-digested walls (b) enriched for peptides were tested.

Fig. 6. Molecular weights of components found in the different HPLC fractions of the solubilized wall. HPLC fractions were analyzed by MS. The molecular weights of their various components were determined and plotted against their retention times. In this experiment, fractions 5, 13, and 20 contained a single component that could be unambiguously analyzed by postsource decay (Table II). The dashed line shows the retention time cut-off above which high TNF-stimulating activity was observed, as depicted in Fig. 4. The many components found at the beginning of the chromatogram indicate that this fraction contained the flow-through.

The MS analysis is presented in Fig. 6. It indicates that most fractions contained more than one component. Moreover, the $M_r$ of these components increased with the retention time, from 600 to 950 in peaks 1–8 (retention time 15–37 min), to 950–1500 in peaks 9–21 (retention time 38–55 min). This MS spectrum was similar when run with five consecutive HPLC separations, three analyzing double-digested walls (step 3A in Fig. 2) and two analyzing peptide-enriched materials (step 3B in Fig. 2).

On one occasion, three fractions (peaks 5, 13, and 20 in Fig. 4) gave a single major ion by MS (Fig. 7), thus allowing further characterization by postsource decay analysis. Table II presents the molecular structures of these components as tentatively deduced from (i) their content in amino acids, (ii) their measured and calculated molecular weights, and (iii) their postsource decay spectrum. Peak 5, which carried a very low inflammatory activity (Table I and Fig. 4), consisted of a dipeptide previously described in the pneumococcal wall (42). Peaks 13 and 20, which carried TNF-releasing activities almost equal to that of LPS (Table I and Fig. 4), consisted of two tripeptides. One of them (peak 20) resembled a previously described structure (43), whereas the other (peak 13) was new.

TNF-releasing Activity of Glycan-enriched Fractions—In the present experiments, the double-digested walls (step 3A in Fig. 2) and the peptide-enriched materials (step 3B in Fig. 2) gave very similar HPLC and MS.

This indicated that the present chromatography conditions preferentially resolved the soluble wall peptides and did not provide much information on the activity of the glycan moiety contained in the original material. To address this question more specifically, the glycan-enriched fraction released during amidase digestion was precipitated as described (step 3B in Fig. 2) and further tested for its TNF-releasing activity before and after digestion with muramidase.

It is noteworthy, however, that this fraction did not only contain the glycan backbone but also some residual peptides that invariably escape detachment by amidase (41). Table III presents the type of material tested and the results of this experiment. First, insoluble and amidase-digested walls had comparable TNF-releasing activities as already described. Sec-
and, the glycan-enriched fraction had an unaltered TNF-releasing activity as compared with insoluble and amidase-digested walls. Third, muramidase-treatment of this glycan-enriched material resulted in a 100-fold decrease in its specific TNF-releasing power. These results indicated a role for the integrity of the glycan backbone to ensure TNF-releasing activity.

**DISCUSSION**

The present results unraveled the existence of very active proinflammatory stem peptides in the pneumococcal peptidoglycan by digesting it with its natural autolytic enzyme amidase. Moreover, quantitative and structural analysis of these stem peptides indicated that a minimal degree of cross-linking between them was necessary to confer TNF-releasing activity. Indeed, one dimeric form of branched peptides (peak 5 with a $M_r$ of 744 in Fig. 3, b and c), which represented a large fraction on the chromatogram, carried no activity. Moreover, material with a lower molecular weight did not carry inflammatory power either. This suggested that simpler structures such as monomeric stem peptides, which also exist in the pneumococcal wall (44), were not inflammatory. This was in agreement with Timmerman et al. (40), who did not detect TNF-stimulating activity with the synthetic monomeric peptide L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine acetate. On the other hand, wall components with a molecular weight greater than 1000 were highly proinflammatory. Several of them had specific (w/w) activities $\geq 100$-fold greater than that of the total wall and almost equal to that of LPS. Two of them were structurally characterized and identified as branched tripeptides.

In the present experiments, the very active wall fragments released by digestion with amidase or digestion with amidase plus muramidase represented only $\approx 2\%$ of the total soluble material. However, this 2% of material was $\approx 100$-fold more active than the 98% of the remaining wall. Therefore, it was tempting to speculate that this 2% fraction comprised the totality of the active wall subcomponents. Although plausible, this hypothesis might greatly oversimplify the reality. Indeed, the wall of Gram-negative bacteria is complex and may contain different types of proinflammatory subcomponents. During enzymatic digestion, it is possible that certain of these inflammatory constituents were destroyed, while others (such as branched stem peptides) were revealed by making them accessible to the target cells.

For instance, Dokter et al. (27) reported that the disaccharide tetrapeptide from *E. coli* peptidoglycan was almost as powerful as LPS as an inducer of interleukin-6 and -1$\beta$ secretion by PBMCs. In the present experiments, we did not find such material resulted in a 100-fold decrease in its specific TNF-releasing power. These results indicated a role for the integrity of the glycan backbone to ensure TNF-releasing activity.

**TABLE II**

| Peak number | $M_r$ (M + H)$^*$ | Amino acid composition | Proposed structure$^{**}$ |
|-------------|-----------------|------------------------|--------------------------|
| 5           | 744.39          | Ala 1.43               | Ala Ala Ala Ala          |
|             | 743.68          | Glx 1                   | Lys                      |
|             |                 | Lys 0.92               |                          |

$^*$ Molar ratio normalized to Glx.

$^{**}$ Based on amino acid composition, molecular weight, and post-source decay analysis.

![FIG. 7. Mass spectra of peaks 5, 13, and 20.](image)

Matrix-assisted laser desorption ionization time of flight mass spectrometry was performed on HPLC fractions corresponding to peaks 1–21. Peaks 5, 13, and 20 were pure as indicated by the presence of a single major ion. Their molecular weights are indicated. Postsource decay analysis was performed on these ions. Their deduced structures are presented in Table II.

**TABLE III**

| Stimulant                        | Putative structures | MSC$_{50}$ (ng/ml)$^*$ |
|----------------------------------|---------------------|------------------------|
| Insoluble cell walls             | ![Structure](image) | 100                    |
| Amidase-solubilized walls        | ![Structure](image) | 100                    |
| Glycan-rich fraction             | ![Structure](image) | 100                    |
| Glycan-rich fraction treated     | ![Structure](image) | 10000                  |

$^*$ The relative stimulatory potency was expressed as the minimum concentration of material required to induce at least a 10-fold increase in TNF levels above background (minimum stimulatory concentration $10^{-6}$ or MSC$_{100}$). Values are means of at least three experiments.

![TABLE III](image)
structures in the pneumococcal wall. However, it is noteworthy that putative pneumococcal disaccharide tetrapeptides could have been destroyed during amidase digestion, because this enzyme hydrolyzes the bonds between \( N \)-acetylmuramic acid and the stem peptide. In this case, they would not have been revealed in the present experiments. Alternatively, it is also possible that disaccharide tetrapeptides of pneumococci were less active than those of \( E. \) coli. Indeed, differences exist between these two constituents of the wall. In many Gram-positive bacteria, such as pneumococci and staphylococci, the third amino acid in the stem peptide is an \( L \)-lysine, whereas this residue is a diaminopimelic acid in \( E. \) coli. Whether this subtle difference might affect the proinflammatory activity of these molecules is as yet undetermined. However, Kengatharan et al. (45) recently reported that the closely related disaccharide pentapeptide of \( S. \) aureus was poorly proinflammatory on its own. A synergism between lipoteichoic acids and disaccharide pentapeptide was required for this staphylococcal component to be active.

The proinflammatory potential of cross-linked peptidoglycan stem peptides has never been tested before. Nonetheless, an indirect clue for the importance of stem peptide cross-linkage may be drawn from previous experiments, utilizing another soluble wall material that held stem peptides close together. This material, so-called soluble peptidoglycan, was released during penicillin treatment of staphylococci (23) and thus consisted of disaccharide-pentapeptide polymers linked together via 1–4 \( \beta \)-glycosidic bonds, but not interpeptide bridges (due to the action of penicillin). Staphylococcal soluble peptidoglycan approached/schematized in Fig. 2 (step 3B). Both of them carried intrinsic inflammatory power after destruction of the glycan chain with muramidase, a process that also disconnected the stem peptides.

In conclusion, the observation that complex peptidoglycan stem peptides have never been tested before. Nonetheless, an indirect clue for the importance of stem peptide cross-linkage may be drawn from previous experiments, utilizing another soluble wall material that held stem peptides close together. This material, so-called soluble peptidoglycan, was released during penicillin treatment of staphylococci (23) and thus consisted of disaccharide-pentapeptide polymers linked together via 1–4 \( \beta \)-glycosidic bonds, but not interpeptide bridges (due to the action of penicillin). Staphylococcal soluble peptidoglycan approached/schematized in Fig. 2 (step 3B). Both of them carried intrinsic inflammatory power after destruction of the glycan chain with muramidase, a process that also disconnected the stem peptides.

While these experiments indicated the importance of glycan chain integrity to ensure a proinflammatory activity, they also might help reconcile previous results with soluble peptidoglycan and the present observations with cross-linked peptides under a common concept. It is possible that in order to stimulate target cells, both soluble peptidoglycan and cross-linked peptides must present at least three stem peptides in close proximity. Below this level of polymerization, peptidoglycan fragments are inactive. Above this level of polymerization, they are active. This hypothesis does not exclude the existence of other inflammatory components in the wall, and/or the existence of components that might interact in synergy with lesser active fragments, as described by Kengatharan et al. (45). However, if true, this hypothesis would provide an important clue as to the structure-activity relationship of some very active constituents of the Gram-positive peptidoglycan. Further studies with synthetic peptides or disaccharide peptide oligomers will be necessary to confirm or disprove this model.

In conclusion, the observation that complex peptidoglycan branched peptides carried high TNF-stimulating activity was novel. The extent of the existence of these peptides occurring freely during infection remains to be seen. Nonetheless, amidase-induced autolysis has been implicated in the pathogenesis of pneumococci (31). The fact that trimeric forms of these peptides were a minimal requirement for activity might provide a clue as to the structural constraint necessary for target cell stimulation. These types of cross-linked peptides are likely to be conserved in the walls of many different Gram-positive pathogens. Moreover, although some intrinsic variation may exist between different bacteria, these cross-linked peptides might be structurally close enough to obey a common stimulatory pathway of target cells. Hence, they might represent a common target for potential anti-inflammatory agents. The existence of such a common stimulatory pathway and of frequently circulating cross-linked peptides in infections is currently under investigation.

Acknowledgments—We are grateful to Professor A. Tomasz for fruitful discussion and M. Knaup for outstanding technical assistance.

---

REFERENCES

1. Vaudaux, P., Grau, G. E., Hugler, E., Schmacher-Perdue, F., Fiedler, F., Waldvogel, F. A., and Lew, D. P. (1992) J. Infect. Dis. 166, 58–64
2. Natansohn, C., Fink, M. P., Hallantan, J. K., MacVittie, T. J., Conklin, J. J., and Parrillo, J. E. (1986) J. Clin. Invest. 78, 250–257
3. Glaser, D. H., Heumann, D., Baumgartner, J. D., and Cohen, J. (1994) Clin. Infect. Dis. 18, Suppl. 2, 205–216
4. Tracey, K. J., Beutler, B., Lowry, S. F., Perryweather, J., Wolpe, S., Milisak, I. W., Hariri, R. J., Fehre, T. J., Zentella, A., and Albert, J. D. (1986) Science 234, 470–474
5. Stevens, D. L., Bryant, A. E., Hackett, S. P., Chang, A., Peer, G., Kosan, S., Emerson, T., and Hinshaw, L. (1996) J. Infect. Dis. 173, 619–626
6. Bone, R. C. (1993) J. Crit. Care 8, 51–59
7. Parsonnet, J. (1989) Rev. Infect. Dis. 11, Suppl. 1, 263–269
8. Crass, R. A., and Bergdoll, M. S. (1986) J. Infect. Dis. 154, 918–926
9. Mattson, E., Verhaeg, L., Roloff, J., Fleer, A., Verhoef, J., and Van Dijk, H. (1993) FEMS Immunol. Med. Microbiol. 10, 261–287
10. Wright, S. D., Hagos, R. A., P. S., Ulevitch, R. J., and Mathison, J. C. (1990) Science 249, 1431–1433
11. Medeiros, T., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997) Nature 388, 394–397
12. Tobias, P. S., Soldau, K., Genniger, J. A., Mintz, D., and Ulevitch, R. J. (1995) J. Biol. Chem. 270, 10482–10488
13. Miethke, T., Wahl, C., Heeg, K., Kechtenbacher, K., Kramer, P. H., and Wagner, H. (1992) J. Exp. Med. 175, 91–98
14. Riesenfeld-Orn, I., Wolpe, S., Garcia-Bustos, J. F., Hoffmann, M. K., and Tsuomanen, E. (1989) Infect. Immun. 57, 1890–1893
15. Spiewasser, T., Miethke, T., Lipford, G., Bierscht, K., Haecker, H., Heeg, K., and Wagner, H. (1997) Nature 386, 336–337
16. Gupta, D., Kirkland, T. N., Viriyakosol, S., and Dziarski, R. (1996) J. Biol. Chem. 271, 23310–23316
17. Casey, L. C., Balk, R. A., and Bone, R. C. (1993) Ann. Intern. Med. 119, 771–778
18. Awada, A., van der Auwera, P., Meanier, F., Daneau, and Klastersky, J. (1990) Clin. Infect. Dis. 15, 53–58
19. Tsuomanen, E., Liu, H., Hengstler, B., Zak, O., and Tomasz, A. (1985) J. Infect. Dis. 151, 859–868
20. Fox, A., Brown, R. R., Anderle, S. K., Chey, C., Cramartie, W. J., Gooder, H., and Schwab, J. H. (1992) Infect. Immun. 59, 1005–1010
21. Dziarski, R., Tapping, R. I., and Tobias, P. S. (1998) J. Biol. Chem. 273, 8680–8690
22. Heumann, D., Barras, C., Severin, A., Glaser, M., and Tomasz, A. (1994) Infect. Immun. 62, 2715–2721
23. Weidemann, B., Brade, H., Rietschel, E. T., Dziarski, R., Bazil, V., Kusumoto, J., Fleid, H. D., and Ulmer, A. J. (1994) Infect. Immun. 62, 478–479
24. Ellum, P. A., Adam, A., Ciobaru, B., and Lederer, E. (1974) Biochem. Biophys. Res. Commun. 59, 1317–1325
25. Dziarski, R. (1991) J. Biol. Chem. 266, 4713–4718
26. Roseman, R. S., and Dziarski, R. (1994) Methods Enzymol. 235, 253–285
27. Dokter, W. H., Dijkstra, A. J., Koopmans, S. B., Stulp, B. K., Keck, W., Halie, M. R., and Vellenga, E. (1994) J. Biol. Chem. 269, 4201–4206
28. Masser, J. L., and Tomasz, A. (1970) J. Biol. Chem. 245, 287–291
29. Holtje, J. V., and Tomasz, A. (1976) J. Biol. Chem. 251, 4199–4207
30. Garcia-Bustos, J. F., and Tomasz, A. (1976) J. Biol. Chem. 251, 33–48
31. Kengatharan, K. M., De Kimpe, S., Robson, C., Foster, S. J., and Thiemermann, C. (1998) J. Exp. Med. 188, 305–315