Role of peptidoglycan subtypes in the pathogenesis of bacterial cell wall arthritis

E Šimelyte, M Rimpiläinen, X Zhang, P Toivanen

Background: Bacterial cell wall (CW) arthritis develops in susceptible strains of rats after a single intraperitoneal injection of the CW from certain bacterial species, both pathogenic and non-pathogenic. For the development of chronic bacterial CW arthritis, the structure of the bacterial peptidoglycan (PG) has been found to be decisive.

Objective: To define the role of PG subtypes in the pathogenesis of chronic bacterial CW arthritis.

Method: Arthritis was induced with CWs of Lactobacillus plantarum, L casei B, L casei C, and L fermentum. Gas chromatography-mass spectrometry was used to measure the presence of PG derived muramic acid in the liver and to determine PG subtypes. CWs were also tested for their resistance to lysozyme in vitro.

Results: These results and those published previously indicate that PGs of CWs which induce chronic arthritis, no matter whether they were derived from strains of Streptococcus, Bifidobacterium, Collinsella, or Lactobacillus, all have lysine as the third amino acid of the PG stem peptide, representing PG subtypes A3α and A4α. Those strains which induce only transient acute arthritis or no arthritis at all do not have lysine in this position, resulting in different PG subtypes.

Conclusions: In vivo degradation of only those PGs with the subtypes A3α and A4α leads to the occurrence of large CW fragments, which persist in tissue and have good proinflammatory ability. CWs with other PG subtypes, even if they are lysozyme resistant, do not cause chronic arthritis, because the released fragments are not phlogistic. It is emphasised that a variety of microbial components not causing inflammation have been found in animal and human synovial tissue.

EXTENDED REPORT

T he cell wall (CW) of Gram positive bacteria has a peptidoglycan (PG) as a major component. PG polymer is composed of alternating N-acetylmuramic acid and N-acetylglucosamine sugar chains with cross-linking peptide chains containing D- and L-amino acids.1 In the CW, polysaccharides (PS) and teichoic acids are covalently bound to PG. These structures in the CW complex, or alone, are biologically active, with immunoadjuvant or slow wave sleep promoting activity.1 During Gram positive infections, PG can activate complement1 and granulocytes,4 and up regulate expression of adhesion molecules on the endothelial cells.1 In vitro, PG is known to induce production of the proinflammatory cytokines interleukin 1, interleukin 6, tumour necrosis factor α (TNFα), and monocyte chemoattractant protein-1 (MCP-1) by monocytes.3,4 Bacterial CWs are digested by a number of PG degrading hydrolytic enzymes, including lysozyme, and the amounts of active CW fragments released depend on the activity of these PG hydrolases.3,5

It has been suggested that PG plays a part in the pathogenesis of chronic inflammation such as rheumatoid arthritis. This hypothesis is based on experimental results obtained after systemic injection of high molecular weight PG-PS complexes, resulting in chronic synovial inflammation. CW components isolated from a variety of Gram positive bacterial species are disease inducing.6 A single injection of bacterial CW causes chronic erosive arthritis1–4 and hepatic granulomas11,15 in susceptible rat strains. On the other hand, purified PG-PS derived from normal human enteric flora can also induce chronic arthritis11,12 and granulomatous enterocolitis12 in the rat. The bacterial species used include Lactobacilli,13,14 Collinsella, Eubacteria15,16 and Bifidobacteria.13,14 It has also seemed that CWs from closely related bacteria within a single genus, including Streptococcus, Lactobacillus, as well as Collinsella and Eubacteria,4,10–21 may be either arthritogenic or non-arthritogenic. Therefore we aimed at determining the factors decisive for the arthritogenicity of the bacterial CW.13,14,16,21,23–26

The differences in arthritogenicity between different CWs seem to depend on the chemical structure of PG.23–26 Variations of the PG peptide moiety occur in the mode of cross linkage, the interpeptide bridge, and the peptide subunit. Based on the anchorage point of the cross linkage to the peptide subunit, the primary structure of PG is divided into type A (cross linkage between position 3 and 4) and type B (cross linkage between position 2 and 4); these are further classified into different subtypes depending on the type/presence of the connecting interpeptide bridges and the amino acid in the third position of the PG peptide subunit.1

In this study we aimed at elucidating the critical role of PG subtypes in the pathogenesis of chronic bacterial CW arthritis. For this purpose, arthritogenicity and concentrations in the liver of the CWs of four Lactobacillus strains were studied in relation to the PG subtypes, and the results obtained were compared with those already published. Additionally, we also evaluated the lysozyme resistance of different PGs.

MATERIALS AND METHODS

Bacterial strains

The following strains of Lactobacillus were used: L plantarum ATCC 4008, L casei C ATCC 25302, L casei B ATCC 11578, and L fermentum ATCC 14931. These strains were purchased from the American Type Culture Collection, Rockville, MD. All bacteria...
were grown by the bioproduction unit in the Department of Biochemistry and Food Chemistry, Turku University.

**CW preparations**

Bacterial CWs were isolated and purified as described previously. To separate 10P (pellet) and 10S (supernatant) fractions, the CW preparations were centrifuged at 10 000g at 4°C for 30 minutes. The protein content determined by the Lowry method in the 10S fractions was 8.0%, 11.6%, 13.1%, and 7.9% per dry weight for *L. plantarum*, *L. casei*, *L. casei* B, and *L. fermentum*, respectively. The total carbohydrate amount determined by the phenol-sulphuric acid method in these preparations was 28.9%, 20.5%, 30.9%, and 18.5% per dry weight, respectively. To exclude endotoxin contamination, the 10S preparations were tested by the E-TOXATE Limulus amebocyte lysate test (Sigma Chemical Co, MO); all tests were negative, with endotoxin concentrations <0.12 EU/ml. The sensitivity of the test was 0.05–0.1 EU/ml. For sterility, the fractions were tested by bacterial culture on agar. The 10S fractions were used in all experiments.

**Chemical structure of L. plantarum CW**

To determine the chemical composition, *P. halotolerans* was isolated from *L. plantarum* according to a previously described protocol. Briefly, the lyophilised 10S fraction of CW was extracted with 10% trichloroacetic acid at 60°C for 4 hours. The suspension was centrifuged at 38 800g at 4°C for 20 minutes, and washed three times with distilled water. The pellet contained the *L. plantarum* PG fraction. The carbohydrates and amino acids of the PG were quantified with the HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with a Hewlett-Packard column, 30 m0.25 mm internal diameter (Hewlett-Packard, Palo Alto, CA), coupled directly to a VG TRIO-1 mass spectrometer (VG Instruments, Manchester, UK) as described previously. The molecules were ionised by the electron impact method and analysed in a selected ion mode using positive ions. Briefly, sugars and amino sugars were analysed as aldito acetate derivatives with mannose and N-methyl-α-glucamine as internal standards, and amino acids as butyl heptafluorobutyl derivatives with norleucine, methionine, and tryptophan as internal standards, respectively. The concentration of the lowest standard used was 2.5 ng.

**Rats**

Pathogen-free inbred female LEW/SsNhsd rats (from the 2028 colony at Harlan Sprague Dawley, Inc, Indianapolis, IN), weighing on average 105 g, were used. The animals were kept in Macrolon III cages with disposable filter tops (Scanbur, Denmark); all handling was performed in a laminar-flow hood. The rats were given autoclaved standard diet and water freely. Before the experiments the animals were allowed to adapt to the local environment for one week. The animal experiments were performed in compliance with national and international laws and policies, and were approved by the Institutional Committee for Animal Research.

**Arthritis induction and clinical evaluation**

Two separate experiments were done: *L. plantarum* and *L. casei* C were used for the first experiment, and *L. casei* B with *L. fermentum* for the second. On day 0, each of the four groups of rats (n = 30–32) was injected intraperitoneally (IP) with sterile phosphate buffered saline (PBS) suspensions of CW preparations (3 ml for each rat). The doses used were chosen on the basis of previously published results (table 1). In the first experiment, with *L. plantarum* and *L. casei* C, the injection dose was 24 mg of CW dry weight per 100 g of rat body weight, whereas in the second experiment with *L. casei* B and *L. fermentum* the dose was 8 mg. Such doses, based on the total carbohydrate concentrations determined in each CW, correspond with a carbohydrate content of 7.3 mg, 5.2 mg, 2.6 mg, and 1.6 mg per rat for *L. plantarum*, *L. casei* C, *L. casei* B, and *L. fermentum*, respectively.

Control rats (n=15) were injected IP with 3 ml of PBS alone. To monitor the development of arthritis, the front and hind paws were scored with a naked eye inspection daily for the first 10 days, and later three times a week. The arthritic symptoms were graded from 0 to 4, based on the degree of erythema, oedema, and functional disorder of the ankle and metatarsal joints (wrist and metacarpal joints), by two independent observers as described previously. The molecules were ionised by the electron impact method and analysed in a selected ion mode using positive ions. Briefly, sugars and amino sugars were analysed as aldito acetate derivatives with mannose and N-methyl-α-glucamine as internal standards, and amino acids as butyl heptafluorobutyl derivatives with norleucine, methionine, and tryptophan as internal standards, respectively. The concentration of the lowest standard used was 2.5 ng.

<table>
<thead>
<tr>
<th>Origin of CW</th>
<th>Dose</th>
<th>Content of</th>
<th>Chronic arthritis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. plantarum</em> ATCC 4008</td>
<td>38.7</td>
<td>Muramic acid†</td>
<td>15.5</td>
<td>–</td>
</tr>
<tr>
<td><em>L. casei</em> C ATCC 25302</td>
<td>42.9</td>
<td>Muramic acid†</td>
<td>14.0</td>
<td>–</td>
</tr>
<tr>
<td><em>L. casei</em> B ATCC 11578</td>
<td>25.0</td>
<td>Muramic acid†</td>
<td>14.0</td>
<td>–</td>
</tr>
<tr>
<td><em>L. fermentum</em> ATCC 14931</td>
<td>21.0</td>
<td>Muramic acid†</td>
<td>20.5</td>
<td>+</td>
</tr>
<tr>
<td><em>C. aerofaciens</em> ATCC 25986</td>
<td>16.7</td>
<td>Total Muramic acid†</td>
<td>28.9</td>
<td>–</td>
</tr>
<tr>
<td><em>C. aerofaciens</em> ATCC 25986</td>
<td>20.0</td>
<td>Total Muramic acid†</td>
<td>20.5</td>
<td>+</td>
</tr>
</tbody>
</table>

ND, not determined.

†Dry weight, per 100 g of rat body weight; †percentage of CW dry weight; †determined on the basis of the present study; †determined on the basis of our previous study; ¶formerly *Eubacterium aerofaciens*.³⁶

**Liver samples**

Four to six rats of each group were killed 1, 3, 7, 14, and 28 days after the CW injection. On each occasion, livers from these and from three control rats were collected. The organs were weighed and stored at –20°C until used. To prepare the liver samples, 5 ml of sterile water was added to each sample before homogenisation with an Ultra Turrax T25 tissue homogeniser (Janke and Kunkel, IKA, Labortechnik, Staufen, Germany).
Germany). The final volume was measured, and the suspensions were analysed by gas chromatography-mass spectrometry (GC-MS) for the muramic acid content. When necessary, liver suspensions were diluted with distilled water to 1:3, 1:5, 1:10, or 1:30. The presence of muramic acid was determined in the whole liver for each rat.

Sample preparation for GC-MS
Muramic acid content in the liver samples was analysed as the trifluoroacetylated methylglycoside derivative by GC-MS, using the chemical ionisation mode with negative ion detection as previously described. To obtain the muramic acid standard for the negative chemical ionisation, E. limosum (ATCC 8486, American Type Culture Collection, Rockville, MD) CW suspension, isolated as described, was used. The muramic acid concentration of the E. limosum CW was determined by GC-MS using the electron impact ionisation as described previously. The standard curve was linear with muramic acid (Sigma Chemical Co, St Louis, MO) concentrations from 10 to 130 ng (injected amount).

For the negative chemical ionisation, samples from liver suspensions and E. limosum CW suspension were evaporated to dryness under a stream of nitrogen at 40°C. The samples were then methanolysed under a nitrogen atmosphere at 85°C for 24 hours in 2 ml of 4 M methanolic hydrochloric acid. Hexane (SupraSolv purity, Merck and Co, Inc, NJ) extraction (3 ml) was performed for the methanolysate. The internal standard N-methyl-D-glucamine (Sigma Chemical Co, St Louis, MO), dissolved in methanol (SupraSolv purity, Merck and Co, Inc, NJ), was dried under nitrogen atmosphere at 40°C and further processed in the same way as the samples. After derivatisation, samples were diluted with 400 µl of toluene (SupraSolv purity, Merck KGaA, Darmstadt, Germany), 50 µl aliquots were put into the sample vials and diluted with derivatised internal standard (50 µl), and 1 µl was injected into the GC-MS system.

Glassware used was first treated with DECON 90 (Decon Laboratories Ltd, Sussex, UK), and 10% DECONEX 11 UNIVERSAL (Borer Chemie Ab, Zuchwil, Switzerland), rinsed with water (2–6 times) between the steps, heated at 170°C for two hours, before autoclaving for 20 minutes.

GC-MS of muramic acid in the liver
The GC-MS analyses were performed with a gas chromatograph (model 6890, Hewlett-Packard, Little Falls, DE) coupled to a mass selective detector (model 5973, Hewlett-Packard, Little Falls, DE) coupled in the splitless injection mode with a slow plunger speed was used. The oven was heated to 210°C, where it was held for one minute. As a post-run step the oven was heated to 300°C for seven minutes. A pulsed helium (purity >99.5%) served as the carrier gas, and isobutane (purity, Merck KGaA, Darmstadt, Germany), 50 µl aliquots were put into the sample vials and diluted with derivatised internal standard (50 µl), and 1 µl was injected into the GC-MS system.

Table 2: Chemical composition of the L. plantarum ATCC 4008 CW. Components of the PG are indicated in bold

<table>
<thead>
<tr>
<th>Component</th>
<th>10S fraction of the CW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates, total</td>
<td>16.5*</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Glucose</td>
<td>0</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Fucose</td>
<td>+</td>
</tr>
<tr>
<td>N-acetylmuramic acid</td>
<td>8.1</td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>8.0</td>
</tr>
<tr>
<td>Amino acids, total</td>
<td>79.5</td>
</tr>
<tr>
<td>Diaminopimelic acid</td>
<td>24.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>19.8</td>
</tr>
<tr>
<td>Glutamic acid/glutamine</td>
<td>12.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.7</td>
</tr>
<tr>
<td>Total</td>
<td>96.0</td>
</tr>
</tbody>
</table>

*Values are expressed as percentage of PG dry weight; †detected, not measured.

The differences between study groups were compared by Mann-Whitney U test for unpaired data and Wilcoxon matched pairs test for paired data. Values of p<0.05 were considered significant.

RESULTS
PG subtypes
Here we determined the PG subtype of L. plantarum ATCC 4008, which was previously unknown. For this purpose the content of carbohydrates and amino acids in the L. plantarum CW preparation was determined. In accordance with the other strains of L. plantarum studied, the results obtained show that the L. plantarum CW used has diamino acidic, alanine and glutamine as PG amino acids, indicating the PG subtype A1γ (table 2; fig 1). The structures of the three other PGs used here are also already known: they are presented in fig 1, with subtype A4α for the other arthritogenic L. casei strains used and the subtype A4β for the non-arthritogenic L. fermentum strain. It should also be noted that L. plantarum does not contain rhamnose (table 2), even though the presence of rhamnose has not been found to be related to the arthritogenicity or non-arthritogenicity of bacterial CWs, in contrast with a previous suggestion.

Arthritogenicity in vivo
To determine the arthritogenicity of the four Lactobacillus CWs used, two separate experiments were carried out. In the first experiment with L. plantarum and L. casei C, rats were injected IP with 24 mg of CW dry weight per 100 g of body weight. This dose of L. casei CW has previously been found to be arthritogenic, and for L. plantarum CW, even higher doses have been reported to be non-arthritogenic. On day 2 after CW injection 5/32 rats had died in the L. casei C group, and 1/31 in the L. plantarum group. Therefore for the second experiment...
with *L. casei* B and *L. fermentum*, a lower dose of 8 mg of CW dry weight per 100 g of body weight was chosen. In the preliminary experiments this dose of *L. casei* B CW was found to be arthritogenic, and for *L. fermentum* CW considerably higher doses were non-arthritogenic[13][19] (table 1). It should also be noted that the amount of muramic acid injected into each rat equalled that previously used to induce chronic CW arthritis with *Collinsella aerofaciens*[21] (table 1).

In contrast with the previous findings indicating the non-arthritogenicity of *L. plantarum* CW,[19] we observed arthritis which lasted for at least 28 days after a single IP injection of *L. plantarum* CW (fig 2). Injection of CWs from *L. casei* C and *L. casei* B also resulted in development of arthritis lasting for at least 28 days, whereas *L. fermentum* induced only a mild acute arthritis, which completely subsided in 10 days; these findings were as expected. Rats injected with PBS alone did not develop any signs of arthritis.

**Muramic acid in the liver**

The presence of CW in the liver was studied on days 1, 3, 7, 14, and 28 by measuring the muramic acid content. This amino sugar is a component of the PG backbone and is not synthesised by mammalian cells. The detection limit of our method was 1 pg (the amount injected into the chromatograph column). Because muramic acid concentrations in the liver are known to reflect those found in the spleen,[8][13] only liver samples were here collected and analysed.

CWs of both *L. casei* (C and B) accumulated in the liver similarly, persisting on day 28 with 20% of the injected muramic acid remaining in the liver, in contrast with the non-arthritogenic *L. fermentum*, which accumulated in the liver only to a small extent and finally cleared away, with <5% of the total dose injected detectable on day 28 (fig 3). Deposition of *L. plantarum* CW was highest on days 3 and 7, and reflected the occurrence of acute arthritis, whereas on days 14 and 28 it was

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**Figure 1** PG structures in the *Lactobacillus* strains used. The *L. plantarum* belongs to the ε-glutamine-ε-diaminopimelic acid-α-alanine subtype (A1γ), the two *L. casei* share the ε-lysine-ε-asparagine subtype (A4α), whereas *L. fermentum* has the subtype ε-ornithine-ε-asparagine (A4β). The enzymes degrading PG cleave specific bonds: lysozyme (β-N-acetylmuraminidase) and N-acetylmuramyl-L-alanine-amidase.

**Figure 2** Development of arthritis in rats injected IP with *Lactobacillus* CW. The injection dose was 24 mg of CW dry weight/100 g of rat body weight for *L. plantarum* ATCC 4008 or *L. casei* ATCC 25302, and 8 mg for *L. casei* ATCC 11578 or *L. fermentum* ATCC 14931. The arthritis score is calculated as a mean value (SEM) for the number of rats indicated at the top. Rats which died on day 2 are excluded from this figure.
To induce chronic arthritis in relation to the PG subtypes, based on the present results and those published by us and by others previously. It appears that PGs of bacterial strains inducing chronic arthritis in the rat, no matter whether they are derived from strains of *Streptococcus*, *Bifidobacterium*, *Collinsella*, or *Lactobacillus*, have lysine as the third amino acid of the PG stem peptide, representing PG subtypes A3α or A4α. On the other hand, all those strains which induce only a transient acute arthritis or no arthritis at all do not have lysine in this position; the position is occupied instead either by ornithine or diaminopimelic acid, resulting in a variety of PG subtypes. Most interesting is a pair of almost identical strains of *Collinsella aerofaciens* (formerly *Eubacterium aerofaciens*), one of them causing chronic arthritis with lysine in the critical position of the PG stem peptide, and the other with ornithine in the same position, and causing only a slight acute, transient arthritis. All these findings strongly suggest that the PG structure with lysine as the third amino acid in the stem peptide is required for induction of chronic bacterial CW arthritis. The only potential exception, which does not fit into the above suggestion, is *L plantarum* CW (table 3). However, the lack of long term tissue accumulation, as seen in the present study, indicates that arthritis caused by this CW cannot be long lasting.

Several previous observations support our suggestion that PG subtypes A3α and A4α with lysine as the third amino acid in the stem peptide are more arthritogenic than other PG subtypes. On the basis of studies on adjuvant arthritis evidence has been presented that structural variations of the chemically defined PG subunits result in drastic changes of the arthritogenic ability, ranging from complete non-arthritogenicity to production of severe arthritis in high frequency. Kohashi et al compared the arthritogenicity of PGs from the CWs of *Staphylococcus aureus* and *L plantarum*. They concluded that a PG subunit with a chain length of two disaccharide units was the minimal structure responsible for arthritogenicity. Consequently, muramyl dipeptide was defined as the minimal arthritogenic structure in adjuvant arthritis. Variation of the third amino acid of the stem peptide has been shown to change the biochemical activity of PG fragments. In studies on subarachnoidal inflammation in the rabbit, PG fragments with lysine in position three were found to be highly inflammatory. Also, branched stem peptides with lysine in position three, isolated from *Streptococcus"


**Pneumococcal PG**, were found to carry TNFα stimulating activity.3 In line with these observations is the demonstration that type A PGs have been reported to be significantly more immunostimulatory than type B PGs.1 4 Similarly, CWs with type A PG activate the complement cascade considerably more effectively than CWs with type B PG.1 4 Furthermore, in our previous study, evidence was obtained to indicate that in strains of *Collinsella aerofaciens*, one of them being arthritogenic with lysine in the critical position and the other being non-arthritogenic with ornithine replacing lysine.2 3 The CW of the arthritogenic strain was a twofold more potent stimulator of the proinflammatory cytokines TNFα and MCP-1 than the non-arthritogenic CW.2 3 After enzyme degradation, the ability of the arthritogenic PG to stimulate production of TNFα and MCP-1 was significantly increased, whereas that of the non-arthritogenic PG was significantly decreased. In other words, after enzyme degradation the arthritogenic PG with lysine in position three had a four- to fivefold stronger stimulatory activity than the enzyme-treated non-arthritogenic PG without lysine in position three.

Table 3 indicates the resistance of different bacterial CWs to lysozyme, in relation to their ability to induce chronic arthritis in the rat. At first sight, it seems that resistance or susceptibility of a CW to lysozyme would bear no correlation to its ability to induce chronic arthritis, even though all CWs carrying chronic arthritis, with the exception of *Streptococcus faecium* have been reported to be lysozyme resistant (table 3). However, it must be emphasised that the arthritis caused by the CW of *Streptococcus faecium* is not as long lasting as that induced by the CW of *Streptococcus pneumoniae*, the former subsiding at two months and the latter being still active at four months after the initial CW injection.16 On the other hand, it also appears that lysozyme resistance is seen among the CWs not causing chronic arthritis (table 3). These seeming inconsistencies can be fully explained, if it is assumed that lysozyme resistant CWs causing longlasting, chronic arthritis accumulate in large quantities in the liver and spleen, known to be the major reservoir after an IP CW injection. This assumption is compatible with the following observations: (a) Lysozyme resistant CWs are degraded in vivo considerably less than the lysozyme susceptible ones, leading to the occurrence of large CW fragments and their longer persistence in the tissues.23 The degree of lysozyme resistance has been found to correlate directly with the amount of CW derived muramic acid found in the tissues,14 23 22 which is also apparent in the present study (fig 3). (b) The liver and spleen act as reservoirs, releasing CW fragments to the circulation and joint tissues.24 25 In the pathogenesis of chronic bacterial CW arthritis the deposition and persistence of CW degradation products in the synovial tissues is crucial.2 4 23 25 However, not all CWs deposited are arthritogenic; a decisive factor is whether the CW fragments released are proinflammatory. Ample evidence exists both from animal15 40–58 and human15–53 studies to indicate that a variety of microbial components are found in the synovial tissues which do not cause inflammation.

On the basis discussed above it is not difficult to accept the possibility that even lysozyme resistant bacterial CWs may be unable to induce chronic arthritis; they represent PG subtypes other than type A3 or A4, and their degradation products are simply not sufficiently proinflammatory, even though they may end up in the joint tissues. In the pathogenesis of chronic bacterial CW arthritis, a PG of an appropriate subtype is required, degradation of which leads to the presence and persistence of phlogistic fragments in the joints, and this seems to be decisive.

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