EXTENDED REPORT

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

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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 CW 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 A3 α and A4 α . 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 A3 α and A4 α 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.

The 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.² During Gram positive infections, PG can activate complement³ and granulocytes,⁴ and up regulate expression of adhesion molecules on the endothelial cells.⁴ In vitro, PG is known to induce production of the proinflammatory cytokines interleukin 1, interleukin 6, tumour necrosis factor α (TNF α), and monocyte chemoattractant protein-1 (MCP-1) by monocytes.^{5–8} 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.^{8–9}

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.¹⁰ A single injection of bacterial CW causes chronic erosive arthritis^{11–14} and hepatic granulomas^{14–15} in susceptible rat strains. On the other hand, purified PG-PS derived from normal human enteric flora can also induce chronic arthritis^{16–17} and granulomatous enterocolitis¹⁸ in the rat. The bacterial species used include *Lactobacilli*,^{13–19} *Collinsella*, *Eubacteria*,^{20–21} and *Bifidobacteria*.^{14–17} It has also seemed that CWs from closely related bacteria within a single genus, including *Streptococcus*,²² *Lactobacillus*,¹⁹ as well as *Collinsella* and *Eubacterium*,^{8–20–21} may be either arthritogenic or non-arthritogenic. Therefore we aimed at determining the

factors decisive for the arthritogenicity of the bacterial CW.^{8–13–14–21–23–26}

The differences in arthritogenicity between different CWs seem to depend on the chemical structure of PG.^{13–21–26} 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

Abbreviations: CW, cell wall; GC-MS, gas chromatography-mass spectrometry; IP, intraperitoneally; MCP, monocyte chemoattractant protein; PBS, phosphate buffered saline; PG, peptidoglycan; PS polysaccharides; TNF α , tumour necrosis factor α

Table 1 Characterisation of CW doses used in studies on bacterial CW arthritis

Origin of CW	Dose			Content of		Chronic arthritis	Reference
	CW (mg)*	Muramic acid (mg)*	Total carbohydrate (mg)*	Muramic acid†	Total carbohydrate†		
<i>L. plantarum</i> ATCC 4008	38.7	ND	6.0	ND	15.5	–	Lehman <i>et al</i> , 1985 ¹⁹
	24.0	1.9	6.9	8.0	28.9	?	Present study
<i>L. casei</i> C ATCC 25302	42.9	ND	6.0	ND	14.0	–	Lehman <i>et al</i> , 1985 ¹⁹
	24.4	1.0	5.0‡	4.0	ND	+	Šimelyte <i>et al</i> , 2000 ¹³
	24.0	1.0§	4.9	ND	20.5	+	Present study
<i>L. casei</i> B ATCC 11578	25.0	ND	6.0	ND	24.0	+	Lehman <i>et al</i> , 1985 ¹⁹
	16.2	0.9	5.0‡	5.3	ND	+	Šimelyte <i>et al</i> , 2000 ¹³
	8.0	0.4§	2.5	ND	30.9	+	Present study
<i>L. fermentum</i> ATCC 14931	21.0	ND	6.0	ND	28.6	–	Lehman <i>et al</i> , 1985 ¹⁹
	27.0	1.6	5.0‡	6.0	ND	–	Šimelyte <i>et al</i> , 2000 ¹³
	8.0	0.5§	1.5	ND	18.5	–	Present study
<i>C. aerofaciens</i> ¶ ATCC 25986	16.7	2.5	ND	15.0	ND	+	Kool <i>et al</i> , 1992 ⁵⁴
	20.0	0.4	11.8	1.9	58.9	+	Zhang <i>et al</i> , 2000 ²¹

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 study¹³; ¶formerly *Eubacterium aerofaciens*.³⁸

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.^{13, 23–27} To separate 10P (pellet) and 10S (supernatant) fractions,²⁸ the CW preparations were centrifuged at 10 000 *g* at 4°C for 30 minutes. The protein content determined by the Lowry method²⁹ in the 10S fractions was 8.0%, 11.6%, 13.1%, and 7.9% per dry weight for *L. plantarum*, *L. casei* C, *L. casei* B, and *L. fermentum*, respectively. The total carbohydrate amount determined by the phenol-sulphuric acid method³⁰ 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 amoebocyte 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, PG was isolated from *L. plantarum* according to a previously described protocol.³¹ Briefly, the lyophilised 10S fraction of CW was extracted with 10% trichloroacetic acid at 60°C for 4 hours. The suspension was centrifuged at 38 800 *g* 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 m×0.25 mm internal diameter (Hewlett-Packard, Palo Alto, CA), coupled directly to a VG TRIO-I mass spectrometer (VG Instruments, Manchester, UK) as described previously.^{13, 21, 26–32} 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 alditol acetate derivatives with mannose and *N*-methyl-D-glucamine as internal standards, and amino acids as butyl heptafluorobutyl derivatives with norleucine, methionine, and tryptophan as internal standards, respectively.³³ The concentration of the lowest standard used was 2.5 ng.

Rats

Pathogen-free inbred female LEW/SsNHsd rats (from the 202B 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.^{13, 21, 23–26} In the rats injected with *L. plantarum* or *L. casei* C the development of arthritis was also monitored by caliper (resolution 0.05 mm; Mitutoyo Asia Pacific Pte Ltd, Japan) measurement of paw swelling; the results were found to agree with those obtained by visual scoring. Rats were killed at different times by cardiac puncture bleeding under methoxyfluorane (Metofane; Pitman-Moore, Inc, Washington Crossing, NJ) anaesthesia.

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). 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.³⁴ 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,²¹ was used. The muramic acid concentration of the *E. limosum* CW was determined by GC-MS using the electron impact ionisation as described previously.³³ 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, Palo Alto, CA). The GC-MS apparatus was equipped with an HP-5MS capillary column (30 m length, 250 µm diameter, 0.25 µm film thickness, Agilent Technologies, Palo Alto, CA). Helium (purity >99.5%) served as the carrier gas, and isobutane (purity 99.5%) as the reagent gas. The starting temperature of the oven (80°C) was held for 2.5 minutes, and programmed at the rate of 8°C/min to a final temperature of 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 splitless injection mode with a slow plunger speed was used with a 2.5 minute pulse time. Selected ion monitoring with negative chemical ionisation and with computerised standardisation, including internal standards, was used for the measurement of muramic acid according to specific guidelines, as described in the Hewlett-Packard G1701AA MS Chemstation workbook (DOS series). A molecular ion with mass/charge ratio (*m/z*) of 567 was used as the target ion, and those with *m/z*=480 and *m/z*=453 were used as qualifier ions. The detection limit was 1 pg, defined as the amount of muramic acid giving a signal to noise ratio of about 20–30 (peak to peak noise). Samples from the same liver suspension were analysed in two to three different runs, always at least in triplicate. The same chemical ionisation mode with a negative ion detection method was used to analyse the muramic acid content in all four *Lactobacillus* 10S fractions.

Degradation of CWs by lysozyme

All the 10S CW fractions from each *Lactobacillus* strain were tested for lysozyme (Sigma, St Louis, MO) sensitivity, as described previously.^{13–35} The assays were performed in triplicate. A CW was considered lysozyme resistant when degradation of the 10S preparation was <30%.

Statistics

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 used was determined. In accordance with the other strains of *L. plantarum* studied,¹ the results obtained show that the *L. plantarum* CW used has diaminopimelic acid, 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^{13–36–37}; 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,^{13–20} in contrast with a previous suggestion.^{16–19}

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,¹³ and for *L. plantarum* CW, even higher doses have been reported to be non-arthritogenic.¹⁹ 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

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

Component	10S fraction of the CW
<i>Carbohydrates, total</i>	16.5*
Rhamnose	<0.4
Glucose	0
Galactose	+†
Mannose	+
Fucose	+
N-acetylmuramic acid	8.1
N-acetylglucosamine	8.0
<i>Amino acids, total</i>	79.5
Diaminopimelic acid	24.8
Alanine	19.8
Glutamic acid/glutamine	12.2
Isoleucine	4.3
Lysine	3.6
Aspartic acid/asparagine	3.4
Threonine	3.1
Valine	2.7
Glycine	2.2
Proline	2.0
Tyrosine	1.4
<i>Total</i>	96.0

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

Figure 1 PG structures in the *Lactobacillus* strains used. The *L. plantarum* belongs to the D-glutamine-m-diaminopimelic acid-D-alanine subtype (A1 γ), the two *L. casei* share the L-lysine-D-asparagine subtype (A4 α), whereas *L. fermentum* has the subtype L-ornithine-D-asparagine (A4 β). The enzymes degrading PG cleave specific bonds: lysozyme (β -N-acetylglucosaminidase) and N-acetylmuramyl-L-alanine-amidase.

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

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* C ATCC 25302, and 8 mg for *L. casei* B 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.

Figure 3 Muramic acid content in rat liver analysed by GC-MS. On day 0 rats were injected IP with CW from *L. plantarum* ATCC 4008, *L. casei* C ATCC 25302, *L. casei* B ATCC 11578, or *L. fermentum* ATCC 14931. The muramic acid levels are given as the mean value (SEM) for four to six rats. Asterisks indicate significant differences ($p < 0.05$) when the rats injected with *L. plantarum*, *L. casei* C or *L. casei* B CWs were compared with those injected with *L. fermentum* CW. Significance is shown separately for each day of analysis.

close to the values of *L. fermentum* CW—that is, close to 0 on day 28. In other words, CWs of the two strains of *L. casei* known to be arthritogenic persisted in the liver, which was not found with the CWs of *L. fermentum* and *L. plantarum*. Livers of the rats injected with PBS alone were found to be muramic acid negative.

Resistance to lysozyme

Incubation for 24 hours with the lysozyme did not produce any degradation of *L. casei* C or *L. casei* B CWs, whereas CWs of *L. fermentum* and *L. plantarum* were lysozyme sensitive, with a 57% and 48% decrease in OD at 560 nm, respectively.

DISCUSSION

Table 3 summarises the ability of different bacterial CWs 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 A3 α or A4 α . 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*³⁸), 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.^{8, 21} 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 A3 α and A4 α 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.^{39, 40} Kohashi *et al* compared the arthritogenicity of PGs from the CWs of *Staphylococcus aureus* and *L. plantarum*.³⁹ 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.⁴⁰ Variation of the third amino acid of the stem peptide has been shown to change the biochemical activity of PG fragments. In studies on subarachnoid inflammation in the rabbit, PG fragments with lysine in position three were found to be highly inflammatory.^{41–43} Also, branched stem peptides with lysine in position three, isolated from *Streptococcus*

Table 3 Arthritogenicity of bacterial CWs in relation to PG subtype and resistance to lysozyme in vitro

Origin of CW	Chronic arthritis in rat			PG structure						Resistance to lysozyme		
	+	/–	Reference	Stem peptide amino acids						+	/–	Reference
				1	2	3	→	4	Subtype			
<i>Lactobacillus casei</i> B ATCC 25303	+		19	Ala	Glu	Lys	Asp	Ala	A4 α	13, 36	+	13, 19
- <i>casei</i> B ATCC 11578	+		19, 27, PS	Ala	Glu	Lys	Asp	Ala	A4 α	1, 13	+	13, 27, PS
- <i>casei</i> C ATCC 25302	+	*	13, PS	Ala	Glu	Lys	Asp	Ala	A4 α	13, 36, 37	+	13, 19, PS
<i>Streptococcus pyogenes</i> D-58 (group A)	+		11, 16, 35	Ala	Glu	Lys	Thr	Ala	A3 α	1	+	16
- <i>faecium</i> ATCC 9790 (group D)	+		16	Ala	Glu	Lys	Asp	Ala	A4 α	1	–	16
- <i>faecium</i> F-24 (group D)	+		16	Ala	Glu	Lys	Asp	Ala	A4 α	1	–	16
<i>Bifidobacterium breve</i> ATCC 15700	+		17	Ala	Glu	Lys	Gly	Ala	A3 α	1	+	17
- <i>adolescentis</i> ATCC 15703	+		14, 17	Ala	Glu	Lys†	Asp	Ala	A4 α	14	+	14
- <i>adolescentis</i> ATCC 15704	+		14	Ala	Glu	Lys†	Asp	Ala	A4 α	1, 14	+	14, 17
<i>Collinsella aerofaciens</i> ‡ ATCC 25986	+		8, 17, 20, 21	Ala	Glu	Lys	Asp	Ala	A4 α	21	+§	8, 26
<i>Collinsella aerofaciens</i> ‡ ATCC 35085	–		8, 21	Ala	Glu	Orn	Asp	Ala	A4 β	21	–	8
<i>Eubacterium limosum</i> ATCC 8486	–		20, 21, 23	Ser	Glu	Orn	Lys	Ala	B2 α	21, 55	–¶	26
- <i>alactolyticum</i> ATCC 17927	–		21	Ala	Glu	Dap		Ala	A1 γ	21, 56	+	25
<i>Lactobacillus fermentum</i> ATCC 14931	–		13, 19, PS	Ala	Glu	Orn	Asp	Ala	A4 β	1, 13	–	13, 19, PS
- <i>plantarum</i> ATCC 4008	?	*	19	Ala	Glu	Dap		Ala	A1 γ	PS	?††	19, PS
- <i>plantarum</i> ATCC 8014	ND			Ala	Glu	Dap		Ala	A1 γ	1	ND	

PS, present study; ND, not determined; →, interpeptide bridge.

*Chronic arthritis was not observed by Lehman *et al*¹⁹; †intraspecies variation to Orn (PG subtype A4 β) has been reported^{1, 14}; ‡formerly *Eubacterium aerofaciens*³⁸; §sensitivity was reported by Severijnen *et al*^{17, 20}; ¶resistance was reported by Severijnen *et al*²⁰; **chronic arthritis was not observed by Lehman *et al*¹⁹ whereas in the present study arthritis lasted for 28 days, with no long term CW accumulation in liver; ††resistance was reported by Lehman *et al*¹⁹ but not seen in the present study.

pneumoniae PG, were found to carry TNF α stimulating activity.⁹ In line with these observations is the demonstration that type A PGs have been reported to be significantly more powerful immunostimulants than type B PGs.^{13,44} Likewise, CWs with type A PG activate the complement cascade considerably more effectively than CWs with type B PG.⁴⁵ Furthermore, in our previous study, evidence was obtained to indicate that in strains of *Bifidobacterium adolescentis*, with intraspecies variation from lysine to ornithine in position three of the stem peptide, a high lysine content is related to the severity of the chronic arthritis.¹⁴

What might be the mechanism by which a single amino acid in a certain position of the PG stem peptide is decisive for the arthritogenicity of a CW? Recently, a direct comparison of the immunostimulatory power of PGs with or without lysine in position three of the stem peptide was carried out using two 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.^{8,21} The CW of the arthritogenic strain was a twofold more potent stimulator of the proinflammatory cytokines TNF α and MCP-1 than the non-arthritogenic CW. After enzyme degradation, the ability of the arthritogenic PG to stimulate production of TNF α 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 ability 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 causing 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 pyogenes*, 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 reservoirs 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.^{22,28} The degree of lysozyme resistance has been found to correlate directly with the amount of CW derived muramic acid found in the tissues,^{14,16,22} 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.^{8,22,46,47} (c) In the pathogenesis of chronic bacterial CW arthritis the deposition and persistence of CW degradation products in the synovial tissues is crucial.^{8,14,22,46,47} However, not all CWs deposited are arthritogenic; a decisive factor is whether the CW fragments released are proinflammatory. Ample evidence exists both from animal^{23,48–50} and human^{51–53} 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 A3 α or A4 α , 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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