Tissue distribution and persistence of arthritogenic and non-arthritogenic *Eubacterium* cell walls

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Abstract

Objective

To study the tissue distribution and persistence of arthritogenic and non-arthritogenic Eubacterium *cell walls (CWs), using arthritogenic* Eubacterium aerofaciens *and non-arthritogenic* Eubacterium limosum.

Methods

Eubacterium aerofaciens *or* Eubacterium limosum *CW was injected into Lewis rats intraperitoneally. Inflammatory changes in the synovium and periarticular tissues were graded histologically. On days 14, 28 and 56 after the injection, the presence of CW in the liver, spleen, mesenteric lymph nodes and synovium was studied by indirect immunofluorescence. In parallel, CW-derived muramic acid in the liver and spleen was measured by gas chromatography-mass spectrometry. In addition, serum TNF-α, IL-1β and IL-10 concentrations were determined by ELISA.*

Results

Systemic injection of Eubacterium aerofaciens *CW, but not of* Eubacterium limosum *CW, resulted in chronic arthritis. Both* E. aerofaciens *and* E. limosum *CWs were observed in the liver and spleen at all of the time points studied. In addition,* Eubacterium limosum *CW was present in non-arthritic synovium on day 14. It was not, however, detected in the synovium or lymph nodes on days 28 and 56, in clear contrast to the rats injected with* E. aerofaciens *CW. According to the analysis by gas chromatography-mass spectrometry, non-arthritogenic* E. limosum *CW had accumulated in the liver cells on days 14 and 28 after the injection to a greater extent than arthritogenic* E. aerofaciens *CW, leading to a lesser distribution in the other organs. A weak trend was observed suggesting that the production of TNF-α and IL-1β, but not of IL-10, is stimulated better by arthritogenic CW than by non-arthritogenic CW.*

Conclusion

Our results indicate that non-arthritogenic CWs are handled by the rat's defence mechanisms in a different way than arthritogenic CWs. The tissue distribution and persistence of CWs play a role in arthritogenicity, but additional factors must exist to determine why the CWs of certain bacteria are arthritogenic and those of others are not.

Key words

Rheumatoid arthritis, *Eubacterium* cell wall, IL-1 , IL-10, muramic acids, inbred LEW rats, TNF- .

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Introduction

A single intraperitoneal (i.p.) injection of group A *Streptococcus pyogenes* cell wall (CW) causes chronic erosive polyarthritis in rats, as first described by Cromartie *et al.* (1). In female Lewis rats severe disease develops with 100% incidence (2), is T cell-dependent (3-6), and is comparable to human rheumatoid arthritis (RA) (7). CWs isolated from human intestinal Gram-positive bacteria can also induce persistent chronic arthritis in this rat model (8-13).

Despite certain structural differences, CWs of all Gram-positive bacteria contain a common polymer, peptidoglycan (PG), which also comprises the major portion of the CW structure and is biologically active both *in vitro* and *in vivo* (13, 14), binding to specific sites on lymphocytes and macrophages (15-17), stimulating macrophages (18), activating alternative complement pathway and inducing sleep (19-21). Bacterial PG consists of a glycan backbone, made up of N-acetylmuramic acid and N-acetylglucosamine, and cross-linked peptide chains containing D- and L-amino acids (22). PG is covalently bound to CW polysaccharide (PS). PG-PS complexes derived from enteric bacterial flora are absorbed from the rat intestine (23, 24), and it has been suggested that the presence of arthritogenic bacteria in resident intestinal flora accounts for the association of arthritis with intestinal inflammation in humans (25).

Several such anaerobic Gram-positive bacterial species have been investigated, including *Eubacterium*, an abundant species in the normal human intestinal flora (26). However, the arthritogenic properties of CW vary between bacterial species within the same genus (9-11, 27), and it is not known what structural characteristics are decisive in this respect. Apparently the development of the chronic disease is dependent on the deposition and persistence of the bacterial CW (9,28-33).

A number of cytokines, such as TNF-α and IL-1β, mediate inflammation and joint destruction in human RA (34, 35) and in experimental animal models (36, 37), including bacterial CW-induced arthritis (38-42). On the other hand, the outcome of immunological activation is regulated by mediators with immunosuppressive and/or anti-inflammatory activity, such as IL-10, which inhibits the secretion of proinflammatory TNF-α and IL-1β (43). Recent reports have demonstrated the down-regulatory effect of systemically administered exogenous IL-10 in experimental allergic encephalomyelitis (44), collagen-induced arthritis (45, 46) and bacterial CW-induced enterocolitis (47).

Based on these findings, we decided to study whether tissue localization and the persistence of CW isolated from the closely related *Eubacterium aerofaciens* and *Eubacterium limosum* species differ, and whether this difference correlates with the ability to induce chronic arthritis. We also examined the potential impact on the development of chronic arthritis of systemic TNF-α, IL-1β and IL-10 expression after *Eubacterium aerofaciens* and *Eubacterium limosum* CW injection.

Materials and methods

Bacterial strains

Eubacterium aerofaciens CCUG 28087 from the Culture Collection of the University of Gothenburg, Sweden, and *Eubacterium limosum* ATCC 8486 from the American Type Culture Collection, Rockville, Maryland, USA, were used.

Isolation of the bacterial CW

Bacterial CW was isolated as previously described (11). In brief, bacteria were cultured in Schaedler broth (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) at 37°C under anaerobic conditions until the late log phase, harvested by centrifugation (10,000 xg, 4°C, 20 min, rotor SS-34) with a Sorvall RC-5C centrifuge (Du Pont Co., Medical Products, Sorvall® Instruments, Wilmington, DE, USA), washed 4 times with PBS (phosphate buffered saline) and heat treated (80°C, 30 min) to kill the bacteria and inactivate the autolytic enzymes (9).

All of the following steps were carried out under sterile conditions. The suspensions were frozen at -20°C and bacteria were disrupted by an X-press (Hydraulic Press HP-20, typex25, AB BIOX, Gothenburg, Sweden). The effectiveness of cell disruption was checked by Gram-

staining. Bacterial CWs were collected by centrifugation (38,700 xg, 4°C, 20 min), re-suspended in PBS and treated with DNase (2 µg/mg wet weight cell wall) and RNase A (20 µg/mg wet weight cell wall) to remove the nucleic acids, washed with PBS and with distilled water, treated with trypsin (20 µg/mg wet weight of cell wall material) and washed as described above.

All enzyme treatments were carried out at 37°C for 120 min, mixing with a magnetic stirrer, and all enzymes were purchased from Sigma, St. Louis, MO, USA. The purified CWs were freeze dried and weighed, re-suspended in 30 ml of PBS (26 mg/ml for *E. aerofaciens* and 52 mg/ml for *E. limosum*) and sonicated for a total of 150 min in an ice bath (Branson Sonifier Cell Disruptor B-15, Smith Kline Co., Danbury, Conn., USA). The CW fragments were separated by centrifugation at 10,000 xg, 4°C for 30 min (48). The pellets and supernatants were separated and labeled as 10P and 10S, repectively. 10S was further ultracentrifuged at 100,000 xg, 4°C for 60 min (Sorvall® Ultracentrifuge, OTD 65B, Ti 60 rotor, Du Pont Co., Medical Products, Wilmington, DE, USA), to obtain 100P (pellet) and 100S (supernatant) fractions. 100P was resuspended in PBS and injected into the rats after a rhamnose assay (49). Before the injection, suspensions of 100P CW fragments were filtered through 0.45 µm pore size filters (Millipore S.A., Molsheim, France) and checked for sterility by bacterial culture.

Animals
Specific pathogen-free female LEW/ SsNHsd rats (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA), from colony 202B, weighing 175 - 195 gr at the beginning of the experiment, 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 an autoclaved standard diet and water *ad libitum*. The animal experiments were performed in compliance with national and international laws and policies (Order No. 1076/85, Finland; EEC Council Directive 86/609, OJL 358, Dec 1987).

Arthritis induction and clinical evaluation
On day zero, 17 rats were injected intraperitoneally with 2 ml of *E. aerofaciens*, and 17 with 3 ml of *E. limosum* CW suspension in PBS (30 µg cell wall rhamnose per 1 gr of body weight). Control rats (n = 6) were given i.p. 2 ml of PBS alone. The animals were observed for 56 days. The front and hind paws were examined by two investigators independently, 2 - 5 times per week. The arthritic symptoms in the rat limb were graded from 0 to 4, based on the degree of erythema, edema, painfulness and functional disorder of the ankle, wrist and metatarsal joints, the total maximum score being 16. Rats were sacrificed by cardiac puncture bleeding under Metofane (Pitman-Moore, Inc., Washington Crossing, NJ, USA) anesthesia at different time points throughout the experiment.

Immunohistology
Fourteen, 28 and 56 days after the CW injection, tissue specimens from the liver, spleen, mesenteric lymph nodes and synovium were collected for immunohistology. Two to 3 rats from both test groups and 1 rat from the control group were sacrificed at each time point. Tissue samples were embedded in O.C.T. compound (Tissue-TEK®, Miles Inc., Elkhart, IN, USA), snap frozen in liquid nitrogen and stored at -70°C. Serial 5 µm sections of frozen tissues were air dried, fixed in 95%/5% ethanol/acetate and stored at -20°C until analysed.
Thawed slides were pre-washed in 0.2% phosphate buffered saline-bovine serum albumin (PBS-BSA) and incubated for 30 min with pre-titrated dilutions of *E. aerofaciens* cell wall-specific monoclonal antibody (mAb) Fu 80-2C1-2-7 (IgG1) and *E. limosum* cell wall-specific mAb Fu 96-5G3-2 (IgG1). Both mAbs were produced in our laboratory by immunizing mice with the respective cell wall preparations (in Freund's adjuvant). Hybridomas from the spleen cells were screened by ELISA for the presence of antibodies specific for *E. aerofaciens* or *E. limosum* CW and were tested by immunofluorescence (IF) for reactivity against phagocytosed bacteria. Further IF studies were carried out to exclude

cross-reactivity with a wide variety of bacteria and normal rat tissues. Secondary antibody, FITC-conjugated anti-mouse Ig F (ab')2 (Silenus Laboratories, Hawthorn, Australia), was diluted 1:100 in 0.2% BSA-PBS containing 10% normal rat serum to block unspecific binding.

As a negative control, all samples were also stained without the primary antibody. Samples from control rats were stained with both CW-specific mAbs and with secondary antibody only. Slides were analysed with a fluorescence microscope by one observer unaware of the origin and the staining of the samples.

Gas chromatography - mass spectrometry
Fourteen, 28 and 56 days after the injection, 3 rats in the *E. aerofaciens* group, 3 in the *E. limosum* group, and 1 rat from the control group were sacrificed, and their livers and spleens were collected and homogenized. The organ suspensions were passed through meshes, and the mononuclear cells were isolated with Lympholyte®-Rat (Cedarlane Laboratories Ltd., Ontario, Canada) gradient centrifugation according to the manufacturer's instructions. Viable mononuclear cells were counted and stored in -70°C until used.
The muramic acid content was analysed as an alditol acetate derivative by gas chromatography-mass spectrometry as described previously (50). Briefly, the samples were hydrolyzed, derivatized and analysed using an HP 5890A gas chromatograph (Hewlett-Packard) equipped with a Noribund SE-54 silica capillary column (Nordion Instruments, Finland) and coupled directly to a VG TRIO-1 mass spectrometer (VG Instruments,UK). The column oven temperature was started at 50°C and programmed to increase at a rate of 10°C/min to 270°C. The molecules were ionized by the electron impact method and analysed in the selected ion monitoring mode using positive ions at m/z = 403 and 445 for muramic acid, and m/z = 327 for N-methyl-D-glucamine as the internal standard (m/z = mass/charge ratio).

Cytokine assays
TNF-, IL-1 and IL-10 levels in rat sera

collected 14 and 28 days after the CW injection were determined using ELISA kits (BioSource International, Camarillo, CA, USA). The sensitivity of the assay was 4 pg/ml for TNF-, 3 pg/ml for IL-1 and 20 pg/ml for IL-10. All measurements were performed in duplicate.

Histology

Specimens of the right ankle from all sacrificed rats were skinned, fixed in formaldehyde stabilized with methanol, decalcified in formic acid, embedded in paraffin and stained with hematoxylin and eosin. Six different criteria (synovial lining/sub-lining cell hyperplasia with periarticular fibrosis, bone destruction, cartilage destruction, inflammatory cell infiltration, fibrin deposits in the joint cavity and villus formation) were evaluated and assigned scores from 0 to 4, the total maximum score being 24 (51).

Statistics

Differences in the arthritis and histopathological scores between the experimental groups were evaluated using Student's two-sided t-test. Serum cytokine levels were compared using the nonparametric Mann-Whitney U test and the Wilcoxon signed rank test. Differences were considered statistically significant if $P < 0.05$.

Results

Clinical and histopathological observations

A single i.p. injection into female Lewis rats of *E. aerofaciens* or *E. limosum* CW was followed by the onset of acute arthritis in the ankle and wrist joints. In the *E. limosum* group the arthritis was extremely mild. The arthritis in the *E. aerofaciens* group culminated on day 3 with a score of 4.44 ± 1.45 (n = 17), whereas in the *E. limosum* group the maximal score was 0.62 ± 0.84 (n = 17) on day 2 (Fig. 1). The incidence of acute arthritis was 100% and 41%, respectively. The development of acute arthritis was accompanied by a loss of body weight (13.5% and 9.2% in the *E. aerofaciens* and *E. limosum* rats, respectively), diarrhea, conjunctivitis, rhinitis and general sickness.
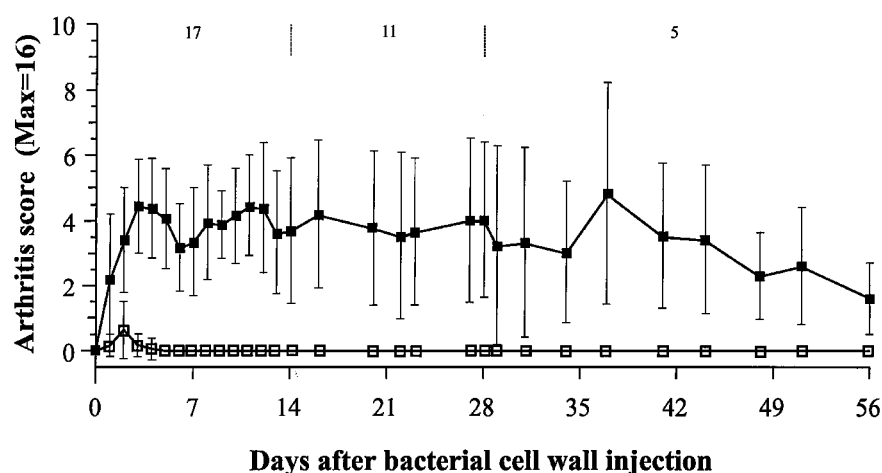Chronic arthritis developed exclusively in the *E. aerofaciens* rats, with a 94%

Fig 1. Clinical course of arthritis in female Lewis/N rats induced by a single i.p. injection of bacterial cell wall from *E. aerofaciens* (■) or *E. limosum* (❏). Mean values ± SD are given; each symbol represents 5 to 17 rats, as indicated by the top line.

Table I. Histopathological evaluation of ankle joints after the injection of cell wall from *E. aerofaciens* or *E. limosum*. Mean ± SD values of the arthritic score (maximum 24) are given. $P < 0.001$, when the *E. aerofaciens*-injected group was compared to the *E. limosum*-injected group.

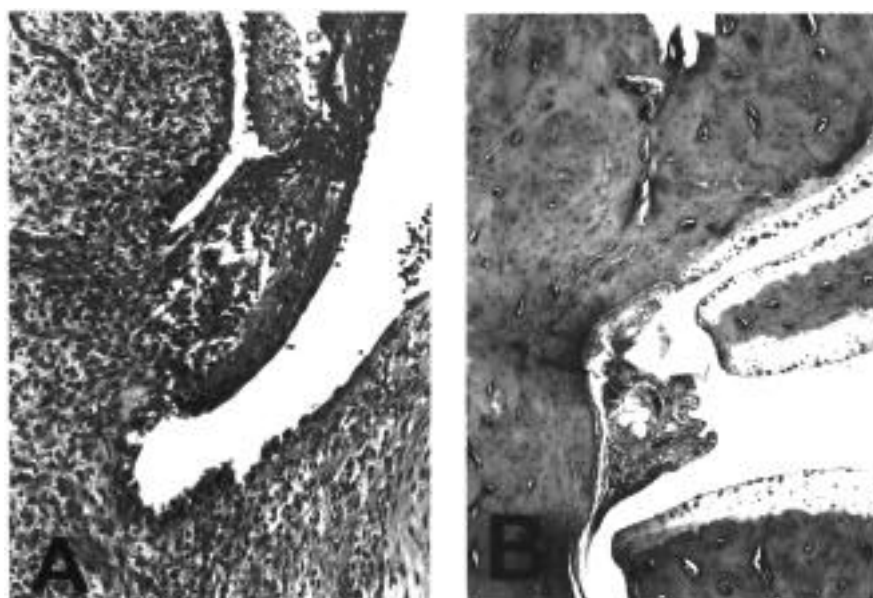| Days after injection	*E. aerofaciens*		*E. limosum*	
14	12.20 ± 1.30	n = 5	1.33 ± 0.82	n = 6
28	12.00 ± 2.83	n = 6	0.83 ± 1.33	n = 6
56	6.20 ± 2.28	n = 5	0.60 ± 0.55	n = 5

Fig 2. Ankle joint histology of a female Lewis/N rat 28 days after i.p. injection of *Eubacterium* cell wall. (**A**) Moderate synovial lining and sub-lining cell hyperplasia, periarticular fibrosis, and inflammatory cells with fibrin deposits in the joint cavity are observed after *E. aerofaciens* cell wall injection (histology score 11/24); (**B**) some synovial thickening, infrequent inflammatory cells and slight villus formation can be seen after injection with *E. limosum* cell wall (histology score 3/24). The clinical arthritis score after 28 days was 4 and 0 for (A) and (B) respectively. Tissue sections were stained with hematoxylin and eosin, magnification x 250.

Table II. Tissue distribution of bacterial cell wall at different time points. Immunofluorescence with mAbs specific for either *E. aerofaciens* cell wall or *E. limosum* cell wall was used.

Days after bacterial cell wall injection	Macroscopic arthritis score	Incidence of chronic arthritis	Liver	Spleen	Lymph node	Synovium
E. aerofaciens						
14	3.68 ± 2.29*	16/17† (94%)	2/2‡	2/2	1/2	2/2
28	4.0 ± 2.43	10/11 (91%)	3/3	3/3	2/3	2/2
56	1.6 ± 1.14	4/5 (80%)	2/2	2/2	1/2	1/3
E. limosum						
14	0	0/17	3/3	3/3	1/3	1/2
28	0	0/11	2/2	2/2	0/2	0/2
56	0	0/5	1/2	2/2	0/2	0/2

* Mean value ± SD
† Number of arthritic rats / total number of rats.
‡ Number of rats positive for the cell wall by IF / total number of rats studied.
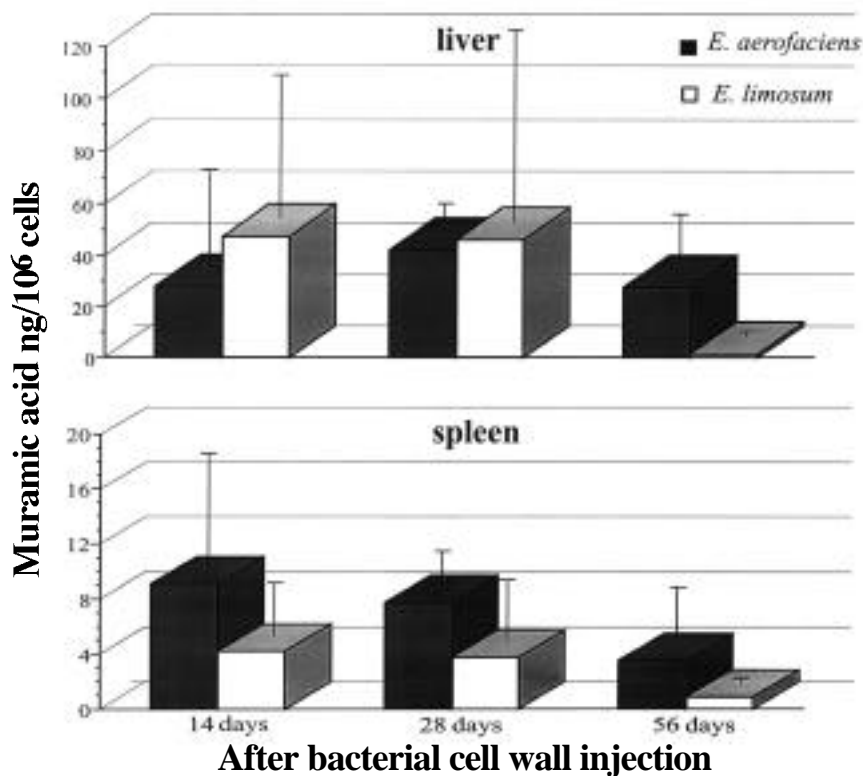
Fig 3. Muramic acid content in mononuclear cells from the liver and spleen, analysed by gas chromatography-mass spectrometry, after the injection of cell wall from *E. aerofaciens* or *E. limosum*. Mean values ± SD for 3 rats are given.

incidence, manifesting in the ankles and to a lesser degree in the wrist joints, with a frequent involvement of the metatarsal joints. The course of the chronic arthritis was fluctuating, with a severity varying from mild to moderate. One rat developed only a very mild arthritis and recovered completely in 23 days. Control rats injected with PBS developed no symptoms (n = 6).

The chronic arthritis in the ankle joints of the *E. aerofaciens* rats was histologically scored at 14, 28 and 56 days after the CW injection (Table I). The inflammatory lesions consisted of moderate to severe periarticular fibrosis, moderate inflammatory cell infiltration and moderate bone destruction, including focal formation of new bone and slight marrow hyperplasia, and were accompanied by focal lessening of cartilage, changes in cartilage matrix staining, sparse fibrin deposition and occasional villus formation (Fig. 2). In addition, at 28 and 56 days a slight thickening of the synovium with occasional inflammatory cells and villus formation was observed in 5/11 *E. limosum* rats, resulting most likely from the acute inflammation.

Immunohistology
Bacterial CW was observed by IF in the liver, spleen, mesenteric lymph nodes and synovium at varying time points after the injection of *E. aerofaciens* or *E. limosum* CW, but not in the PBS-injected control rats (Table II). All specimens from the liver and spleen of rats injected with *E. aerofaciens* or *E. limosum* were positive at each time point, except for one negative *E. limosum* liver sample at 56 days.

In the samples from the lymph nodes and synovium a clear difference between the *E. aerofaciens* and *E. limosum* rats was observed, particularly at 28 and 56 days. At those time points, no evidence of *E. limosum* CW in the lymph nodes or synovium was observed, whereas positivity was seen in the samples from the rats injected with *E. aerofaciens* CW.

Gas chromatography - mass spectrometry
At different time intervals 3 rats were sacrificed from the *E. aerofaciens* and *E. limosum* groups for the determination of the muramic acid content in the liver and spleen; mononuclear cells were isolated for this purpose. The mean values for the muramic acid content in the spleen were always lower by 50% or more in the *E. limosum* injected rats than in the rats injected with *E. aerofaciens* CW (Fig. 3). However, the differences were not statistically significant due to the small number of animals studied (n = 3). In the liver such a consistency was not observed.

Muramic acid was not observed in the liver or spleen samples of rats injected with PBS and studied at the same time points as the other rats; the sensitivity of the method was circa 10 ng of the derivatized muramic acid (50).
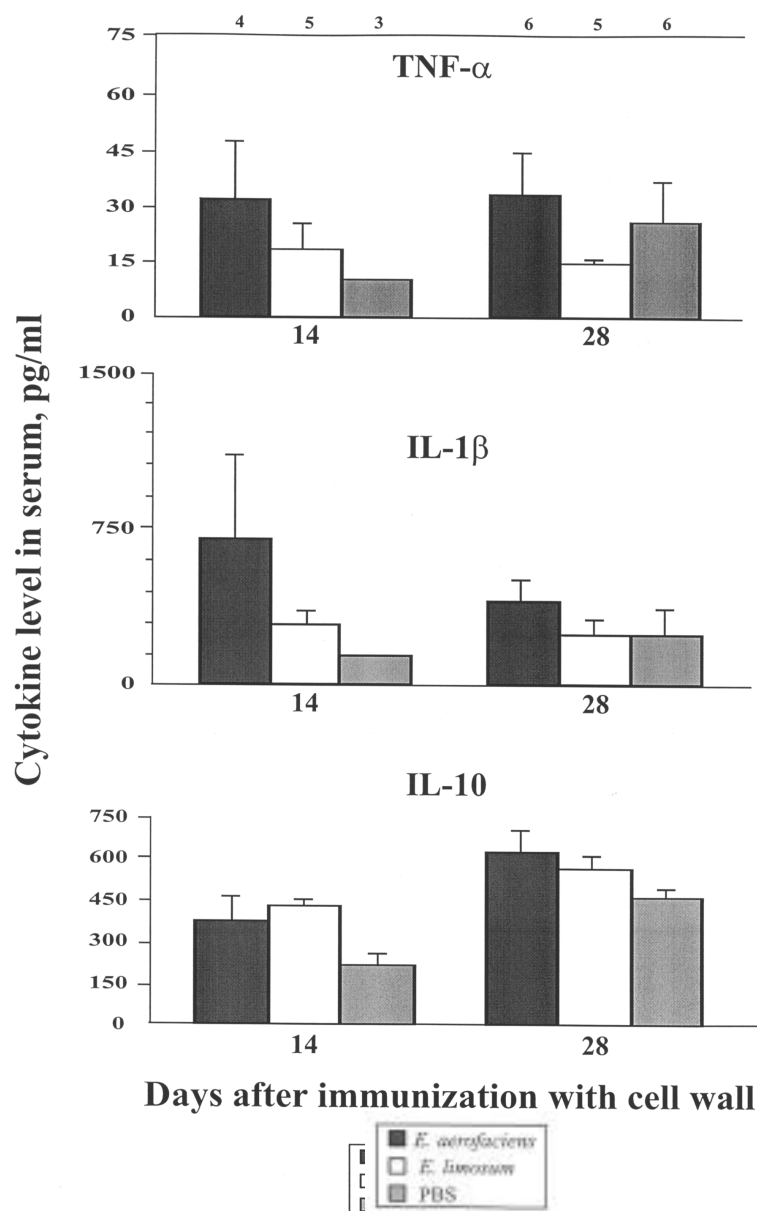
Fig 4. Cytokine levels in sera from female Lewis/N rats injected i.p. on day 0 with *E. aerofaciens* or *E. limosum* cell wall. Each column represents a mean value ± SEM for the number of rats indicated by the top line.

Serum TNF-α, IL-1β and IL-10 levels

Both *E. aerofaciens* and *E. limosum* CWs induced pro-inflammatory and anti-inflammatory cytokine responses 14 days after the immunization (Fig. 4). Slightly higher levels of TNF- and IL-1 were observed in the rats injected with arthritogenic CW than in those injected with non-arthritogenic CW. However, the differences were not statistically significant (P > 0.05). A similar trend could also be seen at 28 days. Regarding IL-10, no evidence for a difference between the two rat groups was obtained at either time point.

Discussion

The aim of this work was to discover what determines the arthritogenicity of CW from *E. aerofaciens* versus the non-arthritogenicity of CW from *E. limosum*. First, on the basis of clinical and histological observations we confirmed that our CW preparations from *E. aerofaciens* and *E. limosum* were arthritogenic and non-arthritogenic, respectively, as described by Severijnen *et al.* (10, 11). Second, it appears that simple presence of bacterial components in the synovial tissue is not sufficient for arthritis production. This has been known for some time;

in 1986 Stimpson *et al.* (52) demonstrated that CWs from non-arthritogenic streptococci were deposited in the rat joint tissue in the same way as were CWs from arthritogenic streptococci. However, in those experiments the non-arthritogenic CW persisted in the joint tissue for up to 45 days, which was not the case in the present study. Unfortunately, our technique for muramic acid quantitation by gas chromatography-mass spectrometry requires sample sizes that were not possible to obtain from the rat synovium. Nevertheless, our immunofluorescence observations are supported by findings indicating that even arthritis-resistant rats accumulate injected bacterial CWs in the joint tissue which cause arthritis in other rat strains (60,30,31). Our results suggest that non-arthritogenic CWs are handled in a different way than arthritogenic CWs. It is possible that non-arthritogenic CWs are cleared more rapidly from the tissues than arthritogenic CWs. This seems to apply particularly to the synovium and lymph nodes. Parallel to our findings, Stimpson *et al.* have reported signifcantly more bacterial material in the liver, both per organ and per gram, after the injection of arthritogenic streptococcal CW than after the injection of non-arthritogenic streptococcal CW (52).

Muramic acid is a structural component that is specific for bacterial CW only (53), and is considered to be an indicator for bacterial material derived from the injection. However, the reports on the presence of muramic acid in normal rat tissues are somewhat controversial. Sen *et al.* (54) detected low molecular weight muramyl peptides extractable in trichloracetic acid in normal rat liver, brain and kidney. Kool *et al.* (55) showed the presence of bacterial antigen in splenic macrophages of normal rats using a mAb specific for a muramic acid-containing epitope. Furthermore, Roten *et al.* (56) have even suggested the possibility of CW PG component synthesis by mammalian cells. We did not observe PG in any of the control animal tissue specimens tested, which is in line with earlier reports (53, 57).

In addition to their tissue distribution, the persistence of CWs might be of importance in the pathogenesis of chronic ar-

thritis (58). Accordingly, resistance to lysozyme is believed to be significant in preventing CW degradation *in vivo* (8, 9, 27). Factors which alter lysozyme activity by modifying the structure of CW also transform the arthritogenicity of CW (48, 59). Furthermore, *in vivo* treatment with mutanolysin, a muralytic enzyme analogous to lysozyme, even prevents chronic disease (60, 61). As reported by Severijnen *et al.* (11), CW isolated from *E. limosum* is resistant to lysozyme, whereas *E. aerofaciens* CW is partially degradable, and our CW preparations confirmed these findings (unpublished observations). However, our results also indicate that *E. limosum* CW is cleared from the synovium and lymph nodes more rapidly than *E. aerofaciens* CW. Therefore, our findings do not support the concept that lysozyme resistance is decisive for arthritogenicity. There may be other, as yet unknown characteristics that determine the persistence of CWs in the tissues, as well as their inflammatory capacity.

In response to the arthritogenic *E. aerofaciens* CW injection, elevated levels of pro-inflammatory TNF- and IL-1 , and decreased levels of anti-inflammatory IL-10 were observed. However, the number of rats studied was small and therefore the difference found was not significant. Surprisingly, we also did not observe any statistically significant differences in the serum IL-10 concentrations between the rats injected with arthritogenic *E. aerofaciens* CW and those injected with non-arthritogenic *E. limosum* CW. The possibility cannot be excluded that various serum factors with an immuno-modulating capacity may have influenced the results. For instance, in human immunoglobulin preparations antibodies to IL-10 have been detected (62). If such antibodies were present in our rat sera, they might have interfered with the IL-10 detection. Published reports on IL-10 serum levels in RA patients are somewhat contrasting, with findings of elevated (63), normal (64, 65) or even decreased (66, 67) serum concentrations of IL-10.

Acknowledgements

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