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Chronic Arthritis Induced in Rats by Cell Wall Fragments of *Eubacterium* Species from the Human Intestinal Flora

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To investigate arthritis-inducing properties of *Eubacterium* species, which are major residents of the human intestinal flora, cell wall fragments (CWF) of several *Eubacterium* strains were prepared and tested in an animal model. After a single intraperitoneal injection in the rat, CWF of *E. aerofaciens*, *E. contortum*, and *E. lentum* induced a chronic polyarthritis. *E. limosum* and *E. tortuosum* CWF induced an acute self-limiting joint inflammation, whereas *E. rectale* CWF failed to do so. The rhamnose contents of the isolated CWF were not related to their arthritis-inducing properties. Paradoxically, the sensitivity of CWF to lysozyme digestion, which is regarded as a parameter for the clearance of CWF in tissues, appeared to be positively correlated with the ability of *Eubacterium* CWF to induce chronic joint inflammation. Our findings show the diversity in arthritis-inducing properties among different species of the anaerobic genus *Eubacterium* and underline the importance of the anaerobic intestinal flora in the induction of joint inflammation.

For several decennia, infectious agents have been implicated in the etiology of rheumatoid arthritis. However, it has not been possible to identify a particular microorganism responsible for the induction of this sterile joint inflammation. Several authors (1, 12, 16, 18, 19) impute an important role to the microbial contents of the bowel; bacterial products or components may pass the bowel wall, especially when the bowel wall has been damaged by infection or inflammation. The continuous influx of bacterial antigens triggers the immune system and may lead to the formation of immune complexes and the deposition of poorly degradable bacterial compounds in joint tissues (20). In susceptible individuals, a distortion of the balance between the influx of bacterial material and the capacity of the body to clear this material may lead to an accumulation of bacterial compounds in tissues, causing inflammation symptoms such as arthritis.

In susceptible rats, a single intraperitoneal injection of cell wall fragments (CWF) from *Streptococcus pyogenes* or *Lactobacillus casei* causes an acute polyarthritis of paw joints followed by a chronic persistent arthritis (5, 14). We have adopted this animal model to study the arthritis-inducing properties of anaerobic intestinal bacteria. Earlier reports (21, 22) showed that CWF of *Eubacterium* and *Bifidobacterium* species have excellent arthritis-inducing properties. *Eubacterium* and *Bifidobacterium* species are gram-positive major residents of the human intestinal flora, occurring in high numbers (up to 10^{10}/g of feces) in the bowels of healthy individuals (2, 17), and thus add considerably to the load of bacterial antigens to which the immune system is exposed. On the other hand, CWF from other bowel flora species, e.g., *Coprococcus*, *Peptostreptococcus*, and *Clostridium* species, fail to elicit chronic arthritis in rats. These large genus-dependent differences in arthritis-inducing properties may also be present between species of a single bacterial genus, as indicated by the profound difference in arthritis-inducing properties of *E. aerofaciens* and *E. rectale* CWF, the latter failing to give any joint inflammation symptoms upon intraperitoneal inoculation.

Differences in arthritis-inducing properties might be related to the biochemical composition of the bacterial cell wall, especially its rhamnose contents (15, 26); also, the in vitro resistance of bacterial CWF to digestion by lysozyme has been related to its ability to induce a chronic arthritis (15, 27). We have found that the relationships between arthritis-inducing properties of CWF isolated from several anaerobic bacterial species and their lysozyme sensitivity and rhamnose contents are not as clear-cut as was concluded from experiments in which mainly *Streptococcus* and *Lactobacillus* CWF had been investigated (15, 26, 27). As the CWF described in our previous paper (22) originated from several anaerobic bacterial genera, this heterogeneity may conceal the clear relationship found when modified streptococcal CWF were studied (27). So, we have isolated and studied CWF from several strictly anaerobic species belonging only to the genus *Eubacterium*; the rhamnose contents as well as the susceptibility to in vitro lysozyme digestion of the CWF were investigated in relation to the ability of the CWF preparations to induce chronic arthritis after a single intraperitoneal injection.

MATERIALS AND METHODS

Animals. Female Lewis rats (Harlan Sprague Dawley, Bicester, Oxfordshire, United Kingdom) weighing 109 to 197 g were used throughout the study. Groups of four or five rats were injected intraperitoneally with an aqueous suspension of the *Eubacterium* CWF (5). A cell wall dose of 25 μg of muramic acid per g of body weight was given; control rats were injected with an equal volume of phosphate-buffered saline. The animals were observed for the development of paw inflammation at regular intervals during 60 days; diameters of wrists and ankles at the distal end of the radius and at the malleoli, respectively, were measured with a vernier caliper five times in weeks 1 and 2, three times in weeks 3 and 4, two times in weeks 5 and 6, and one time in weeks 7 to 9.

Bacteria. *E. aerofaciens* ATCC 25986, *E. contortum* ATCC 25540, *E. lentum* ATCC 25559, *E. tortuosum* ATCC 25548, and *E. limosum* ATCC 8486 were obtained from the American Type Culture Collection, Rockville, Md.; *E. rectale* was isolated from the fecal flora of a healthy individual and was identified according to Holdeman et al. (11). All

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TABLE 1. *Eubacterium* cell wall dose and resulting paw diameter increase

Source of CWF (*n*)[a]	Amt of cell wall dose constituent[b]				Increase of sum paw diam (mm) during phase:	
	Muramic acid	Dry wt	Rhamnose	Total carbohydrate	Acute	Chronic
E. aerofaciens A (5)	25	304	95	269	3.98	8.06
E. aerofaciens B (5)	25	153	47	125	3.91	4.77
E. contortum (5)	25	141	44	167	3.24	3.36
E. lentum (5)	25	164	59	117	0.99	3.66
E. tortuosum (4)	25	184	10	ND[c]	5.30	1.70
E. limosum (4)	25	124	74	140	1.39	1.12
E. rectale (5)	25	130	2	33	−0.17	−0.17
Control (5)					0.68	−0.15

[a] No. of rats injected.
[b] Micrograms per gram of body weight.
[c] Not determined.

strains were cultured overnight at 37°C on Schaedler broth (Oxoid Ltd., London, England) under strictly anaerobic conditions after inoculation with a log-phase culture. For *E. lentum*, the culture medium was supplemented with 5 g of arginine per liter (25).

Preparation of cell wall fragments. Bacterial CWF were prepared as described by Cromartie et al. (5), followed by the differential centrifugation procedure of Fox et al. (8). Briefly, cells were harvested, washed, and fragmented with glass beads in a Braun shaker (Melsungen). Cell walls were collected by centrifugation (10,000 × *g*), treated with ribonuclease and trypsin, washed, and sonicated (Measuring and Scientific Equipment, Ltd., Crawley, United Kingdom) for 75 min. After sedimentation of debris, the sonicated cell wall suspension was centrifuged at 10,000 × *g* for 30 min.; the 10,000 × *g* supernatant was centrifuged two times at 100,000 × *g* for 60 min. Both 100,000 × *g* pellets were collected, suspended in phosphate-buffered saline, and used for intraperitoneal injection after passage through a 0.45-μm-pore-size membrane filter (Millipore Corp.) and subsequent control for sterility. Two CWF preparations of the *E. aerofaciens* strain were made (Table 1), one with (A) and one without (B) the differential centrifugation procedure, by using the 10,000 × *g* supernatant after enzyme digestion and sonication.

Chemical analysis of cell wall preparations. Muramic acid and rhamnose contents were determined as described by Hadzija (10) and Dische and Shettles (6), respectively. The total amount of carbohydrate was determined according to Dubois et al. (7), with galactose as the standard.

Lysozyme digestion of bacterial CWF. CWF suspensions were diluted in 0.1 M sodium acetate buffer, pH 5.0, to an A_{560} of about 0.8. Per milligram of dry weight of CWF, 0.1 mg of lysozyme (egg white; Sigma Chemical Co., St. Louis, Mo.) was added, and during incubation at 37°C, the A_{560} was measured at regular intervals (27). As a control, CWF suspensions were incubated at 37°C without the addition of lysozyme.

Statistical analysis. The mean increase of the sum of the paw diameters (sum paw diameter) from days 1 to 15 and days 16 to 60 after cell wall inoculation, compared with the sum paw diameter at the day of inoculation, was taken as a parameter for the severity of the acute and chronic arthritis, respectively. For each rat injected with the low CWF dose, the mean increases of sum paw diameter during the acute and chronic phases of the observation period were calculated; subsequently, the median value of these mean increases of sum paw diameter was calculated for each group of rats. The correlations between the median increases of the sum paw diameter and the doses of CWF, rhamnose, and total carbohydrate and the lysozyme sensitivity in the seven groups of rats were evaluated for both the acute and chronic phases of arthritis by using the Spearman rank correlation test. To evaluate the increase of sum paw diameter in each rat group, the mean values of sum paw diameter of each rat during the acute and chronic phases of the observation period were compared with the values on day 0 by using the paired *t* test.

Histology. After 60 days, rats were sacrificed by cardiac-puncture bleeding under ether anesthesia. Skinned-ankle joint specimens were fixed in 1:10 (vol/vol) diluted buffered 36% formaldehyde solution, decalcified in 5% (vol/vol) formic acid for 5 days, and embedded in paraffin. Specimens of liver tissue were fixed in the diluted formaldehyde solution. Sections were stained with hematoxylin and eosin.

RESULTS

Arthritis induction by CWF from *Eubacterium* species. Table 1 lists the dose of *Eubacterium* cell walls given intraperitoneally and shows the resulting changes in sum paw diameter during the acute and chronic phases of the observation period. In general, the *Eubacterium* species differed greatly in their arthritis-inducing properties (Table 1; Fig. 1). CWF from some strains induced a persistent chronic arthritis of paw joints, whereas CWF from other strains failed to do so. The changes of the paw diameter during both phases after the cell wall injection were significant ($P < 0.05$ [paired *t* test]) except in the *E. rectale* CWF rat group and in the control rats. *E. aerofaciens* A CWF, obtained after differential centrifugation, induced a more progressive chronic arthritis than *E. aerofaciens* B CWF, prepared without the additional centrifugation steps ($P = 0.01$ [Wilcoxon test]); results obtained with the *E. aerofaciens* B cell walls have already been presented (22). *E. contortum* CWF induced an acute arthritis followed by a chronic joint inflammation; not all of the animals were affected to the same extent. Rats inoculated with *E. lentum* CWF showed a late start of the chronic phase of joint inflammation. At day 60, the livers of these rats appeared to be covered with numerous small tumors (see below). These *E. lentum* CWF-injected rats showed general malaise during the whole observation period, whereas rats inoculated with other *Eubacterium* CWF showed malaise only during the first days after cell wall injection. CWF of *E. tortuosum* and *E. limosum* induced a self-limiting acute polyarthritis, whereas *E. rectale* CWF did not elicit joint inflammation symptoms in the inoculated rats. No mortality was seen after *Eubacterium* CWF injection. Control rats did not show macroscopic signs of paw inflammation.

Biochemical composition of isolated CWF. The muramic acid contents of the isolated *Eubacterium* CWF varied from 8 to 20% of the dry weight (Table 2); thus, peptidoglycan was the major cell wall component of the isolated cell walls. The rhamnose contents showed a greater range: in *E. rectale* CWF, rhamnose was almost absent, whereas 59% of the *E. limosum* CWF dry weight was rhamnose. Table 2 also lists the amount of total carbohydrate; because these results are based on galactose as the standard, a carbohydrate which may not be representative of the *Eubacterium* cell wall carbohydrate, the actual amount may be overestimated.

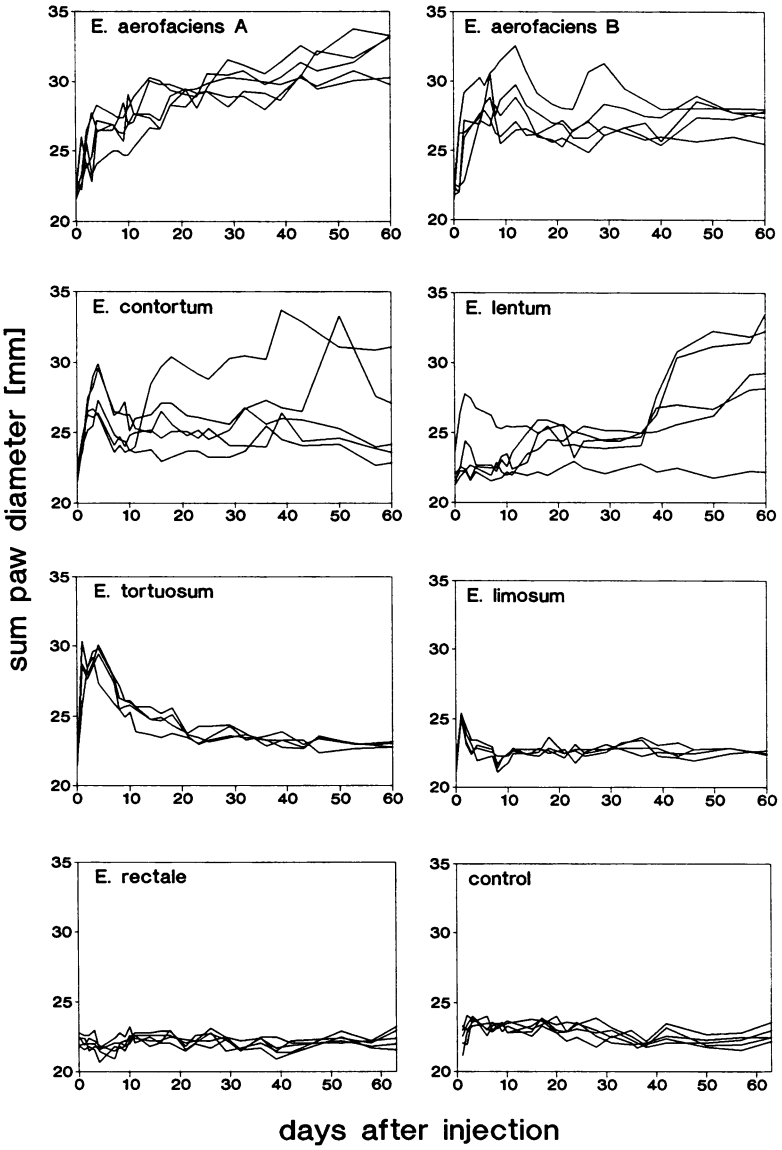

FIG. 1. Inflammation induced in rat paws by the intraperitoneal injection of *Eubacterium* CWF; each line represents a single rat. At day 0, rats were injected with a CWF dose of 25 μg of muramic acid per g of body weight. The *E. aerofaciens* A and B CWF were prepared with and without the differential centrifugation procedure, respectively.

Table 1 shows the actual dose of cell wall components in the *Eubacterium* strains injected and gives the severity of the joint inflammation, expressed as increase of paw diameter during the acute and chronic phases of arthritis. When the severity of the chronic arthritis, represented by the median value of sum paw diameter during days 16 to 60 of the observation period, was compared with the rhamnose dose given to the seven rat groups, no significant correlation was found ($r = 0.61$; $P = 0.16$). Neither was there a significant correlation between acute arthritis and the rhamnose dose ($r = 0.18$; $P = 0.71$) nor between the total carbohydrate dose and the severity of the chronic joint inflammation ($r = 0.65$; $P = 0.18$).

Lysozyme sensitivity of *Eubacterium* cell wall preparations. The percent decrease in A_{560} after 8 h of incubation with lysozyme (Table 2) shows a major variance in the sensitivity of *Eubacterium* species to digestion by lysozyme. *E. lentum*, *E. limosum*, and *E. rectale* CWF were not digested, whereas

TABLE 2. Biochemical composition and lysozyme sensitivity of *Eubacterium* CWF

Source of CWF	Biochemical composition of CWF[a]			Lysozyme sensitivity[b]
	Muramic acid	Rhamnose	Total carbohydrate	
E. aerofaciens A	8	31	88	69
E. aerofaciens B	16	31	82	31
E. contortum	18	31	118	44
E. lentum	15	36	71	1
E. tortuosum	14	5	ND[c]	8
E. limosum	20	59	112	0
E. rectale	19	2	25	0

[a] % Dry weight.
[b] % Decrease of the optical density at 560 nm after 8 h of incubation with lysozyme compared with the optical density at 560 nm at time 0.
[c] Not determined.

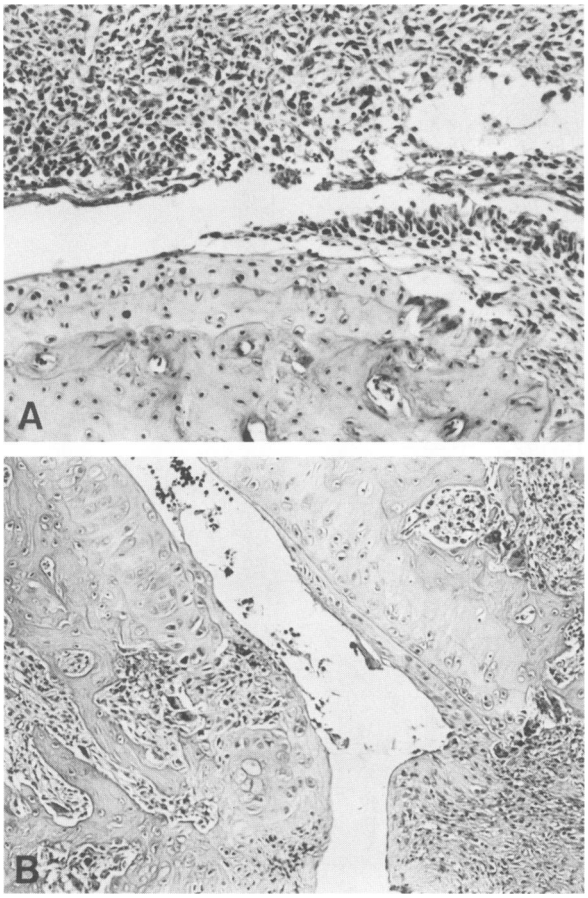

FIG. 2. Histological appearance of tissue inflammation induced by *Eubacterium* CWF 60 days after intraperitoneal injection. (A) Section through a hind paw of a rat injected with *E. aerofaciens* CWF. Heavy infiltration of subsynovial tissue with lymphocytes, histiocytes, and some polymorphonuclear cells is markedly present. Also a marginal cartilage erosion with overlying pannus tissue can be seen. Magnification, ×334. (B) Section through a hind paw of a rat injected with *E. lentum* CWF. Infiltration of subsynovial tissue with inflammatory cells and some synovial fibrosis are seen. Marginal cartilage erosions with osteoclasts are present, with pannus tissue invading the joint cavity. Some inflammatory cells are present in the joint space. Magnification, ×298.

E. aerofaciens and *E. contortum* were clearly sensitive to lysozyme. *E. tortuosum* CWF were digested to a small degree. Incubation of CWF suspensions at 37°C without lysozyme added showed no decrease in A_{560}, except for the *E. aerofaciens* B CWF suspension, which gave a 14% decrease in the A_{560} after 8 h of incubation. No correlation was found when the sensitivity of the *Eubacterium* CWF to digestion by lysozyme was compared with their biochemical composition. A significant correlation was present between the sensitivity of the *Eubacterium* CWF and the severity of the chronic joint inflammation ($r = 0.81$; $P = 0.02$); regarding the acute arthritis, the relationship was not significant ($r = 0.71$; $P = 0.08$).

Histology. Hind-paw sections of a rat sacrificed 60 days after inoculation with *E. aerofaciens* A CWF showed active joint inflammation with swollen synovial-lining cells and infiltration of subsynovial tissue with polymorphonuclear cells, lymphocytes, and histiocytes (Fig. 2A). The deeper layers of subsynovial tissue expressed a fibrous reaction, with edema, infiltration by lymphocytes, and vascular proliferation. Heavily infiltrated spots alternated with areas without almost any inflammation, a phenomenon also seen in joint sections of rats inoculated with other *Eubacterium* CWF. Some marginal cartilage erosions were seen near infiltrated synovia. Periosteal apposition of new bone tissue was prominent. In ankle joints of rats injected with *E. aerofaciens* B CWF, no active inflammation foci were observed, but there was marked apposition of new bone and one of the major ankle joint spaces was partly replaced by connective tissue, resulting in joint ankylosis. Subsynovial tissue showed fibrosis; polymorphonuclear cells and lymphocytes were absent. Sections through an ankle joint of an *E. contortum* CWF-inoculated rat showed gross disturbance of bone and joint architecture, with a marked apposition of new bone and an increased turnover of bone with the presence of multiple osteoclasts. No cartilage or synovial tissue was found; probably this had been replaced by connective tissue, as was seen between metatarsal bones.

Ankle joint sections of an *E. lentum* CWF-inoculated rat showed extensive joint inflammation: subsynovial infiltrations with mononuclear and polymorphonuclear cells, fibroblasts, numerous synoviocytes (30), histiocytes, vascular proliferation, and some marginal cartilage erosions (Fig. 2B). Some joint spaces contained polymorphonuclear cells. Extensive apposition of new bone was present, with an increased bone turnover characterized by the presence of numerous osteoclasts. Some periarticular inflammatory foci were observed, mainly consisting of polymorphonuclear cells. Liver sections of this rat showed numerous circumscribed fibrous granulomatous lesions; the sometimes necrotic centers consisted of polymorphonuclear cells and were surrounded by mononuclear cells, fibroblasts, and histiocytes. The granulomas were distributed in a metastatic pattern. Multinucleated giant cells were seen in a minority of the granulomas.

Joint sections of *E. tortuosum* CWF-injected rats showed signs of an extinguished inflammation: active inflammation foci were absent, while subsynovial tissue showed some fibrosis with an increase of small blood vessels; synovial-lining cells were only slightly swollen; and no bone or cartilage changes were seen.

Ankle joint sections of rats inoculated with *E. limosum* CWF showed comparable, but milder, synovial abnormalities.

Neither joint sections of *E. rectale* CWF-inoculated rats nor those of control rats showed any pathology.

DISCUSSION

Species of the genus *Eubacterium* dominate the gram-positive part of the human obligate anaerobic intestinal flora, representing up to 16% of the total bowel flora (2, 17, 28); this means that the number of *Eubacterium* species is 6×10^9/g of bowel content. This study shows that CWF from three of the six *Eubacterium* species investigated, i.e., *E. aerofaciens*, *E. contortum*, and *E. lentum*, induced a persisting chronic arthritis. Recently, Benno et al. (2) showed that these strains were present in the fecal floras of healthy individuals, i.e., *E. aerofaciens*, *E. contortum*, and *E. lentum* in 29 of 30, 3 of 30, and 8 of 30 individuals, respectively; so, the selected strains are normal intestinal flora bacteria.

CWF from the *Eubacterium* species showed a spectrum of arthritis-inducing properties, ranging from nonarthritis inducing (*E. rectale*) and self-limiting acute arthritis inducing

(*E. tortuosum* and *E. limosum*) to persistent chronic polyarthritis inducing (*E. aerofaciens*, *E. lentum*, and *E. contortum*). The observation that *E. tortuosum* and *E. limosum* CWF induce a self-limiting acute joint inflammation proves that an initial acute joint damage is not necessarily followed by a chronic phase of joint inflammation. Histological sections of hind-paw joints from *E. limosum*- and *E. tortuosum*-inoculated rats made after the waning of the acute joint inflammation show synovial fibrosis, so the acute joint swelling must be based on a synovitis and is not merely a periarticular soft-tissue swelling. *E. rectale* CWF prepared without the differential centrifugation procedure lacked arthritis-inducing properties (22); the additional centrifugation steps did not improve its arthritogenicity. Our findings underline the relevance of intestinal flora bacteria in the etiology of arthritis and extend previous observations that the large bowel harbors bacteria capable of inducing a wide spectrum of arthritis-inducing properties.

According to histological examination of joint sections made 60 days after *Eubacterium* cell wall injection, three joint inflammation patterns can be distinguished. First, an active joint inflammation was still present at day 60, with infiltration of synovial tissue and reactive changes in bone and joint structure, as seen in rats injected with *E. aerofaciens* A and *E. lentum* CWF. Second, a subsided synovial inflammation was visible, with gross proliferative changes in bone and joint structures. Obviously, the prolonged acute joint inflammation was extinguished, whereas the reactive bone destruction was still present, as observed in *E. aerofaciens* B- and *E. contortum*-injected animals. Third, after a self-limiting acute joint inflammation, minimal residual synovial abnormalities were still found, while bone and cartilage had a normal aspect, as can be seen in *E. limosum*- and *E. tortuosum*-injected animals. Thus, the arthritis patterns observed by measuring paw diameters (Fig. 1) agree with histological findings.

It is known that CWF from *S. pyogenes* (29) and *Bifidobacterium breve* (22) are capable of inducing liver granulomas after a single intraperitoneal injection. Our finding that *E. lentum* CWF are also able to induce these liver granulomas emphasizes the ability of CWF from anaerobic intestinal flora bacteria to induce a prolonged inflammation as a result of CWF persistence in tissues.

Our previous report (22) shows that CWF from several bacterial genera differ greatly in arthritis-inducing properties. Here we show that, when several bacterial strains from a single genus are examined, a similar variety in arthritis-inducing properties can be seen. Arthritis- and nonarthritis-inducing species may even occur within a single bacterial genus, as has been indicated by the differences in arthritis-inducing properties between CWF of the two *E. contortum* strains isolated from the floras of patients with Crohn's disease (21).

To clarify the profound differences in arthritis-inducing properties between *Eubacterium* species, we analyzed cell wall characteristics relevant to the bacterial cell wall arthritis model. As peptidoglycan is the essential arthritis-inducing cell wall component (4, 5, 8), we chose to establish our cell wall preparations on the basis of their contents of muramic acid, the characteristic building block of the amino-sugar backbone of peptidoglycan. Several other cell wall factors are supposed to determine the arthritis-inducing property, i.e., CWF size (8), the integrity of the peptidoglycan polysaccharide complex (4, 5), the rhamnose contents of the cell wall (15, 26), and the resistance of the cell wall to in vitro digestion by lysozyme (14, 27). Because our CWF isolation procedure results in intact peptidoglycan polysaccharide complexes with a limited spread in fragment size, we paid special attention to the variable factors, rhamnose contents, and lysozyme sensitivity.

Stimpson et al. (26) and Lehman et al. (15) observed a striking relationship between the rhamnose contents of bacterial CWF and their ability to induce a chronic arthritis upon intraperitoneal injection. Our previous observations with CWF from bacteria of different genera followed the suggested trend but could not give significant support to their findings (22). When CWF from several bacterial strains of the genus *Eubacterium* were tested, the correlation between rhamnose dose and severity of chronic arthritis appeared not to be significant. This indicates that rhamnose is not the major factor contributing to the arthritis-inducing property of a given bacterial cell wall but is, together with lysozyme resistance, one of several cell wall properties which determine the joint inflammation properties of bacterial CWF.

Stimpson et al. (27) found that the resistance of bacterial CWF to lysozyme is crucial for the ability of CWF to induce a chronic joint inflammation; in their study, they used chemically modified *S. pyogenes* CWF. In another study from Stimpson et al. (26), however, lysozyme-resistant CWF from *Peptostreptococcus productus* failed to induce chronic arthritis whereas *Streptococcus faecium* CWF, which were sensitive to lysozyme, did induce a chronic joint inflammation. Lehman et al. (15) showed that CWF from *Lactobacillus plantarum* and *Lactobacillus fermentum*, which were 20 and 97% degradable by lysozyme, respectively, and lysozyme-resistant CWF from *L. casei* did not induce chronic arthritis, whereas moderately degradable (5%) CWF from another *L. casei* strain induced severe chronic arthritis. In an earlier report on the arthritis-inducing properties of CWF from several anaerobic bacteria (22), we already showed that the relationship between the ability to induce a chronic arthritis and lysozyme resistance is not clear-cut. The lysozyme sensitivity of *Eubacterium* CWF, as given in Table 2 of the present study, demonstrates that CWF with good arthritis-inducing properties were degradable by lysozyme, whereas CWF with poor or absent arthritis-inducing capacities were resistant to lysozyme.

We tried to fit the data discussed above into one general conclusion: a complete insensitivity or high sensitivity of CWF to lysozyme is related to an inability to induce chronic joint inflammation; a moderate sensitivity of CWF to lysozyme is related to the ability to induce arthritis. The present results agree with these conclusions.

In the adjuvant-induced-arthritis model, it has been demonstrated that minimal changes in the peptidoglycan oligopeptide chains result in profound changes in arthritis-inducing activity (3, 13). Information about these *Eubacterium* cell wall characteristics, which might be also relevant for the CWF arthritis model we used, is not available. Chemical analysis of the peptidoglycan structure of three *Eubacterium* species by Guinand et al. (9) and Severin et al. (23, 24) shows great heterogeneity, which also might be present among the *Eubacterium* species described in this paper. In our laboratory, work is in progress to investigate peptidoglycan structure in more detail to relate bacterial cell wall composition to arthritis-inducing properties.

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