HUMAN INTESTINAL FLORA AND THE INDUCTION OF CHRONIC ARTHRITIS - STUDIES IN AN ANIMAL MODEL

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HUMAN INTESTINAL FLORA AND THE INDUCTION OF CHRONIC ARTHRITIS - STUDIES IN AN ANIMAL MODEL

DE HUMANE DARMFLORA EN DE INDUCTIE VAN CHRONISCHE GEWRICHTSONTSTEKINGEN - STUDIES IN EEN DIERMODEL

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CHAPTER 1

ARTHRITIS AND INTESTINAL FLORA

The etiology of rheumatoid arthritis (RA), a chronic joint inflammation, is unknown. A microbial involvement is suspected, but no particular microorganism has been incriminated. The human intestinal microflora is an abundant and continuous source of bacterial antigens and may be involved in the induction or maintenance of chronic joint inflammation.

In this introductory chapter I shall describe the possible relationship between joint inflammation, bowel flora and intestinal disease. Clinical syndromes which suggest this relationship are presented. Characteristics of the human intestinal flora are described, together with bacterial cell wall structures and their immunological properties. Several aspects of the hypothesis that intestinal bacterial antigens may pass the intestinal wall and give rise to inflammatory symptoms at distant locations are discussed. Animal models developed to study joint inflammation, including two models which use bacterial antigens to elicit arthritis, are presented. Finally, the experimental work of this thesis is introduced.

1.1 RHEUMATOID ARTHRITIS AND MICROORGANISMS

Rheumatoid arthritis is a chronic disease in which a sterile joint inflammation is the most striking feature. About 1-2% of the human population is affected; it is a major cause of disability. One or several joints (hand, elbow, knee, foot, ankle, hip) may be affected, showing redness, swelling, pain, tenderness and morning stiffness, ultimately leading to joint deformation. The disease is systemic and present in multiple organs, but is expressed most apparently in the joints. The presentation of the disease is often insidious, but an acute attack of joint inflammation may also be the beginning of a protracted illness. Exacerbations and remissions are often seen. The onset of RA is most frequent during the third and fourth decade. The disease is more often seen in women than in men (ratio 3:1). An increased frequency of the HLA-DR4 antigen has been found in affected persons [1-3].

Histologically, a vasculitis of the joint synovium is a hallmark symptom of early

RA, followed by synovial infiltration with inflammatory cells and synovial proliferation. This leads to pannus formation, destruction of marginal cartilage and adjacent bone tissue, as can be seen on X-rays of affected joints. The disease may be accompanied by subcutaneous rheumatoid noduli and systemic manifestations of vasculitis, e.g. pulmonary fibrosis. Rheumatoid factor (antibodies against the Fc portion of IgG) can be detected in the serum of the majority of RA patients [1-3].

The etiology of RA still remains obscure, but a microbial involvement has been suspected for several decades [4-6]. Several microorganisms have been incriminated but no claim has stood the test of time [5-11].

Arguments for an infectious etiology of RA are several:

(a) septic arthritis has clinical similarities with RA;
(b) some forms of sterile non-RA joint inflammations are related to infection elsewhere in the body;
(c) in several animal models arthritis can be induced by bacterial cell wall antigens;
(d) bacterial cell wall compounds have potent inflammatory properties;
(e) RA patients show increased levels of serum antibodies against bacterial cell wall structures.

Examples of microorganisms which have been incriminated in RA include difteroids [12], mycoplasmata [13,14], *Clostridium perfringens* [9,15], mycobacteria [15-18], several viruses such as parvovirus and Epstein-Barr virus [19], but these claims for etiological involvement appeared not to be convincing.

The failure to pinpoint a particular microorganism as causative in RA caused a shift in the attention of researchers from an exogenous source of microbial antigens towards an endogenous one. The abundant resident microflora of the large bowel may well account for a continuous release of immunopotent bacterial antigens into the *milieu intérieur*, resulting in induction or maintenance of chronic joint inflammation [6-8,10,11,20-22]. It is supposed that bacterial fragments can pass the bowel wall, especially when the bowel wall is damaged by infection or inflammation. The immune system is exposed to these microbial substances, and immune complexes containing bacterial antigens [6] will be formed which are transposed to joints and other organs and give rise to inflammatory symptoms. It is known that the peptidoglycan part of Gram-positive bacteria is poorly biodegradable: streptococcal cell walls are protected from *in vivo* digestion by means of their polysaccharides [23]. Therefore, after entrance into the milieu intérieur, bacterial debris may persist in tissues and contribute to chronicity of the inflammation. Distortion of the delicate balance between influx of bacterial antigens and their removal by the immune system may lead to accumulation of bacterial products in tissues [20,24].

The joint synovium has some special features which may explain why the joints are preferentially affected after systemic administration of microbial antigens. The synovium is highly vascularized, which is necessary for the nourishment and cleaning of the avascular cartilage. The absence of a basement membrane under the synovial lining

[25] might contribute to an intensified spread of bacterial antigens which arrive via synovial vessels. As discussed by Schulz *et al.* [24], there are several areas in the joint where highly vascularized tissues are adjacent to avascular tissues nourished by diffusion, e.g. perichondrium, peritendineum, periosteum and endo- and perimysium. The borderline between vascular and avascular tissues is supposed to be prone to antigen deposition, and there is ineffective antigen elimination due to an insufficient inflammatory response of avascular tissue. These borderline localizations correspond with predilection sites for chronic rheumatoid inflammation in man and animals. The cartilage matrix may also act as a kind of reservoir in which antigens are dumped and from which they can be released [10,26]; liberation of antigens may lead to a flare of the joint disease.

1.2 JOINT INFLAMMATION AND BOWEL DISEASE

Bowel-related microbial etiology in RA is suggested by clinical and laboratory observations in a series of sterile joint inflammation diseases:

(a) About 5-23% of patients with inflammatory bowel diseases (IBD), e.g. Crohn's disease (CD) and ulcerative colitis (UC) suffer from attacks of joint inflammation, especially of the major limb joints. The severity of the joint inflammations often parallels the activity of the intestinal disease; arthritis occurs more frequently in CD when the colon is affected [27]. Unlike RA, this bowel disease-associated joint inflammation rarely leads to erosions and irreversible joint damage; rheumatoid factor is usually negative [28-30]. In UC, colectomy results in complete remission of the arthritis. Surgical therapy for CD results in remission of arthritis in only 50% of cases [11]. The peripheral arthritis is not correlated with the HLA-B27 genotype whereas the spondylitis and sacroiliitis, which also may complicate IBD, are positively linked to HLA-B27 [11]. In contrast to peripheral arthritis, the axial arthritis is not alleviated by bowel surgery [29,30].

(b) In reactive arthritis, an acute, sterile oligoarthritis develops days to weeks after an infection at a remote site [31]. This is seen in Reiter's disease, in which an urethritis, probably caused by *Chlamydia trachomatis* or *Ureoplasma urealyticum* is complicated by a sterile arthritis and conjunctivitis [32]. The arthritis which complicates bowel infection by *Salmonella*, *Shigella* or *Yersinia* species is another example of reactive arthritis. In most cases, major joints of the extremities are affected. Viable microorganisms could not be demonstrated in synovial fluid. The majority of the patients are HLA-B27 positive. Molecular mimicry between bacterial surface antigens and antigenic determinants on host cells is assumed to be an underlying mechanism [31,32]. In two recent studies, Granfors *et al.* [33,34] showed the presence of *Yersinia* or *Salmonella* antigens in synovial cells of patients with reactive arthritis. Ileocolonoscopy showed macroscopic abnormalities in 14 of 33 (HLA-B27 positive and negative) patients with idiopathic reactive arthritis [35]; a microscopic bowel wall inflammation was present in

31 of 33 patients.

(c) Patients treated for morbid obesity by a jejunoileal bypass show, in 5-35% of the cases, an arthralgia or arthritis of limb and small peripheral joints [36,37]. The arthritis is transient and recurrent, and neither erosive nor deforming [38]. Rheumatoid factor usually is negative. Bacterial overgrowth of the blind loop and the small bowel is supposed to contribute to the joint inflammation [39,40]. Immune complexes containing antibodies against faecal flora antigens (*Escherichia coli*, *Bacteroides fragilis*) and complement components are found in serum of patients suffering from an acute attack of arthritis following intestinal bypass surgery [41]. Antimicrobial treatment results in improvement of the joint symptoms [37,39]. When the arthritis is otherwise untreatable, reconstruction of the continuity of the bowel leads to a rapid relief of joint inflammation symptoms [36,38].

(d) Ankylosing spondylitis (AS), a progressive stiffness of the lumbar vertebrate column, occurring predominantly in young male adults, is associated with the presence of HLA-B27 [42]. Investigators have shown a relationship with faecal carriage of *Klebsiella* in patients with active spondylitis [43], but these findings could not be confirmed by other groups [44]. Ebringer *et al.* [45] demonstrated an increased serum antibody titer against *Klebsiella pneumoniae var oxytoca* in patients with active spondylitis. Also, an increase in serum antibodies against a peptidoglycan moiety (D-Ala-D-Ala) has been demonstrated in AS patients [46]. Several controversies exist regarding the mechanisms by which the immunological response to *Klebsiella* antigens may lead to disease, as has been lucidly reviewed by Keat [44]. Patients with AS and peripheral arthritis also show microscopic bowel wall inflammation upon ileocolonoscopy. AS patients without peripheral arthritis showed less severe bowel wall inflammation [35,47,48]. AS patients also showed increased uptake of labeled ethylenediaminetetraacetic acid (EDTA) after oral ingestion, suggesting an increase in bowel wall permeability [49].

These clinical pictures demonstrate that, in some forms of sterile joint inflammation, a (bowel-related) microbial origin is strongly suspected. As has been stated, a microbial etiology has been suspected in RA for several decades. In 1922 the American surgeon Rea Smith reported on the beneficial effect of partial colectomies and ileosigmoidostomies carried out in patients suffering from 'arthritis deformans' to remove 'intestinal foci of infection' [50]. As of 1922 Crohn's disease had not yet been described as a separate disease entity, part of the above patients might in fact have been CD patients. This is supported by the observation that radiological abnormalities in the ileocecal region were seen in part of the patients described by Smith.

An attractive feature of the hypothesis that the bacterial load originating from the bowel lumen is the initiating factor in RA is that not a single microorganism, but the microbial load as a whole, has to be responsible, taking into account that the microbial components must be presented in sufficient amounts [51]. A bacterial component common to all bacteria, i.e. the bacterial cell wall peptidoglycan, is a serious candidate

for causing joint inflammation symptoms. The permanent exposure of the immune system to these bowel derived antigens may account for chronicity of the disease. Clinical associations between arthritis and bowel inflammation or infection as shown above, together with findings in some animal models for arthritis (to be discussed in a following section), support this hypothesis.

1.3 THE INDIGENOUS INTESTINAL FLORA AND RA

With regard to the involvement of bacterial constituents from the intestinal contents in RA, several items need to be discussed; each one may contribute to the etiology of RA:

(a) The bowel flora may be altered in RA.
(b) In the bowel lumen of RA patients bacteria may disintegrate more than in healthy subjects (not experimentally supported).
(c) The bowel wall of RA patients may be more permeable.
(d) The immune system of RA patients may differ in its response to bowel-derived microbial constituents.
(e) Joints of RA patients may be more prone to an immune response to microbial compounds than joints of healthy subjects (HS).

1.3.1 The intestinal flora of healthy individuals and RA patients

The human indigenous bowel flora is characterized by the presence of very high numbers of bacteria in the colon and the terminal ileum. More than 30% of the faecal mass is bacteria with up to 2×10^{11} bacteria per gram faeces (wet weight), with > 99% of them being strictly anaerobic [52]. About 50% of the intestinal bacteria are Gram-positive; of these bacteria, peptidoglycan is the major cell wall component making up 30-70% of the cell wall [53]. Another hallmark of the human intestinal flora is the huge variety in bacterial genera and species. It is estimated that up to 400 anaerobic bacterial species may be present in the faecal flora of a single individual [52].

Major Gram-positive residents of the human intestinal flora are members of the genera *Eubacterium*, *Bifidobacterium*, *Clostridium*, *Lactobacillus* and *Peptostreptococcus*, each present in numbers exceeding 10^9 per gram faeces wet weight. The major Gram-negative genera are *Bacteroides* and *Fusobacterium* [54]. The flora of the terminal ileum is less numerous and less heterogeneous than in the colon, with $10^{3.5}$ to $10^{6.3}$ bacteria/ml bowel contents [54]. The flora of the jejunum and the proximal ileum is even less numerous and is made up by aerobes and a minority of anaerobes [54,55].

At birth, the bowel contents are sterile, but shortly afterwards *E. coli* and *Streptococcus* species are found and within 4-6 days nearly all full-term, vaginally-

delivered infants are colonized with the anaerobes *Bacteroides*, *Clostridium* and *Bifidobacterium*. A major change in bowel flora occurs when the infant is weaned and when it gradually gets adjusted to 'adult' food. By the end of the second year the intestinal flora of a single individual reaches its definite composition [56] and remains stable for years [57]. No major changes in bowel flora composition occur with increasing age [58,59].

As has been demonstrated by van de Merwe *et al.*, the composition of the faecal flora is under genetic influence: a study in twin pair children showed that the faecal flora of monozygotic twins are much more alike than those of dizygotic twins [60]. Patients with CD and their (a-symptomatic) children share an abnormal bowel flora, characterized by elevated numbers of *Bacteroides* species and the presence of Gram-positive coccoid rods [61].

The intestinal flora is important in the maintenance of colonization resistance [62], i.e. the presence of an indigenous flora impedes colonization by pathogens. The intestinal flora has a major influence on the structure and function of the gastrointestinal tract [63,64], plays an important role in shaping the developing immune system [64,65] and contributes to the metabolic activity of the digestive system [55,66].

Little is known about the composition of the bowel flora in RA patients, especially with regard to anaerobic bacteria. Olhagen and Månsson [15,67] demonstrated an increased number of *C. perfringens* in the faecal flora of RA patients. This observation was confirmed by Shinebaum *et al.* [68]. Differences in other bacterial species (*E. coli*, *Klebsiella*, *Shigella*, *Salmonella*, *Yersinia*, *Pseudomonas*, *Proteus*, *Streptococcus faecalis*), however, were not found. Total numbers of aerobes and anaerobes did not differ between RA and HS. Other research groups could not confirm the increased number of *C. perfringens* in the intestine of RA patients [69,70]. So far, investigators refrained from investigating the composition of the anaerobic intestinal flora, neglecting the majority of the intestinal bacteria. A recent report indicated that the jejunal flora may be altered in RA. With the help of jejunal aspirate cultures and glucose hydrogen and ^{14}C-glycocholate breath tests an abnormal jejunal flora was detected in 5 out of 17 RA patients [71]. Taken together, there is some experimental evidence that supports the hypothesis that in RA the bowel flora is altered.

1.3.2 Structure and immunologic properties of peptidoglycan

The bacterial cell wall owes its strength to peptidoglycan (PG). Basically, the PG structure is the same in Gram-positive and Gram-negative bacteria. It is a large biopolymer, made up of long amino sugar chains, cross-linked by oligopeptides (Fig. 1, left). The amino sugar chains consist of alternating N-acetylmuramic acid and N-acetyl-D-glucosamine, coupled by ß-1,4 bonds. The carboxyl group of muramic acid is substituted by an oligopeptide which is directly, or via an interpeptide bridge, cross-linked to adjacent peptide subunits [53,72]. This peptide moiety contains alternating L-

and D-amino acids (Fig. 1, right). In Gram-positive bacteria, a thick, three-dimensional network of PG is constructed around the cell membrane. The composition of the amino sugar strand is almost identical in various bacteria, but the composition of the oligopeptide chains varies between species. These differences found between several genera and species have led to a taxonomic grouping [53].

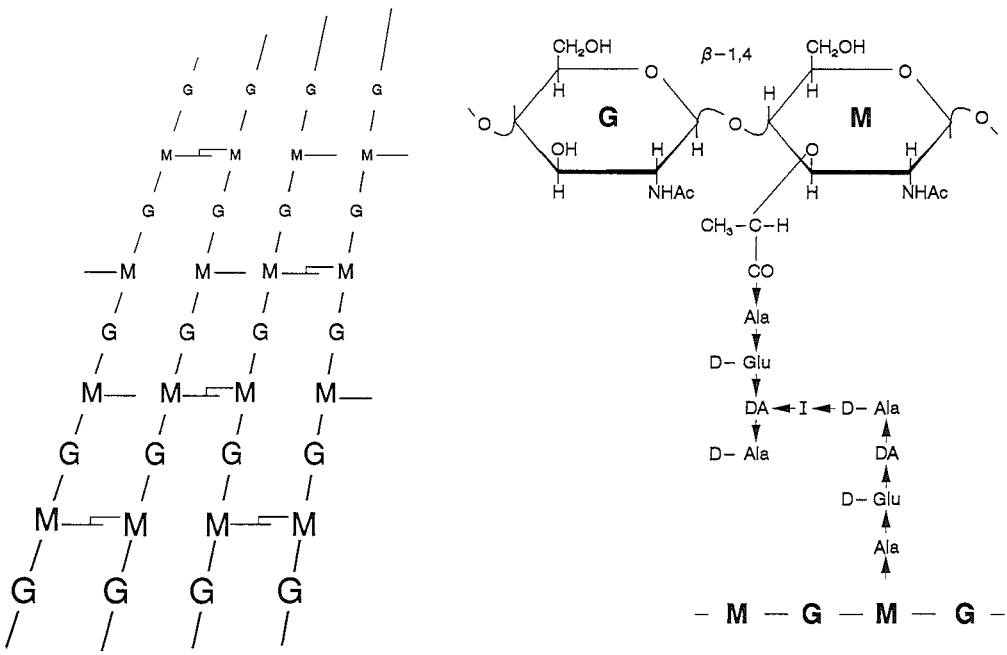

Figure 1. (left) structure of a peptidoglycan monolayer: chains of amino sugars, linked by oligopeptides; (right) primary structure of a typical peptidoglycan fragment, made up by two amino sugar groups with oligopeptide side chains, linked by an interpeptide bridge.
Abbreviations: Ac: acetyl; DA: D-diamino acid; G: N-acetylglucosamine; I: interpeptide bridge; M: N-acetylmuramic acid.

On the outer part of Gram-positive bacteria, the PG is covered by a polysaccharide layer covalently coupled to it, protecting the bacterial cell wall against enzymatic breakdown by lysozyme and digestion by host leukocytes [23,73]. In *S. pyogenes*, the polysaccharide part consists of polyrhamnose chains, with N-acetyl-glucosamine attached to it [73,74]. Of most other bacteria, especially the anaerobes, the composition of the polysaccharide layer is not known. Some PG constituents are unique for bacteria and are not found elsewhere, e.g muramic acid and D-alanine [53].

In Gram-negative bacteria the PG layer is less thick and less cross-linked by oligopeptide side chains than in Gram-positive microorganisms. PG accounts for less

than 10% of the Gram-negative cell wall [53] but may account for up to 70% of the cell wall in Gram-positive bacteria and is built up by 30-40 layers of amino sugar chains.

PG has several potent immunological properties, as has been reviewed by Stewart-Tull [75] and Heymer *et al.* [7]. Its biological activities have been reviewed by Dziarski [76]. PG activates complement by the alternate and classical pathways [77,78], and stimulates macrophages [76,79] leading to interleukin 1 (IL-1) production [80]. B-cells are polyclonally stimulated by PG [81-83]. The injection of PG in animals leads to the production of auto-antibodies e.g. rheumatoid factor, probably as a result of polyclonal stimulation of B lymphocytes [84]. The *in vitro* production of rheumatoid factor by PG-stimulated lymphocytes has also been reported [85]. As suggested by Ginsburg [20], macrophages might fail to digest bacterial fragments after uptake; the release of partly degraded antigens by macrophages [86] may lead to a perpetual inflammation. Bare PG is quickly degraded *in vivo* [18,23], in contrast to PG covalently linked to polysaccharide which may persist in tissues for months [23,87,88]. The immunodominant part of PG with regard to antibody production is the peptide moiety; the amino sugar part is crucial for its adjuvant and arthropathic properties [72].

Tubercle bacilli ground in mineral oil potentiate the immune response to a protein component in water which has been subsequently emulsified in the mineral oil [89]. This adjuvant effect is exhibited by a fraction of the mycobacterial cell wall. However, mycobacteria can also be replaced by PG complexes of several other bacteria [90] or even muramyldipeptide [91,92].

In addition to these capacities PG can also induce arthritis in rats, as discussed in section 1.4.

1.3.3 The uptake of bacterial antigens from the bowel lumen

Translocation

Intestinal bacteria are associated with the bowel wall: the mucus layer which covers the epithelial lining cells is colonized by bacteria, but bacteria probably do not adhere to the bowel wall [93]. This bowel wall-associated flora contains species which are also found in the bowel contents, but it is not clear whether or not the composition of this bowel-associated flora differs from that of the bowel contents. The passage of viable bacteria from the gastrointestinal tract through the epithelial mucosa to mesenterial lymph nodes is called translocation. As anaerobic bacteria seldomly translocate through an unaffected bowel wall [94] and (in the rabbit) indigenous anaerobic bacteria do not adhere *in vitro* to caecal epithelium [95], the uptake of whole anaerobic microorganisms through the bowel wall is much less than that of aerobic bacteria [94].

Regarding bacterial cell wall fragments (CWF), which occur in the bowel contents in considerable quantities [96], the situation might be fully different. Small bacterial products liberated during bacterial growth and division [97-100] may more easily pass the bowel wall and spread to tissues. The presence of bacterial cell wall remnants in

mammalian tissues has been demonstrated elegantly by Sen and Karnovsky [101]. They detected muramic acid in liver, kidney and brain tissue of naive rats. The presence of anti-peptidoglycan antibodies in the serum of patients with RA also indicates exposure to bacterial antigens.

Bowel wall permeability in RA

Experimental results regarding bowel wall permeability in RA do not support the hypothesis that the bowel wall is more permeable in RA. Reports in which low-molecular weight test molecules, ^{51}Cr-EDTA and polyethylene glycol (PEG) (M=400-1000), are used, show that intestinal permeability probably is not increased in RA [102-105]. In RA patients on non-steroid anti-inflammatory drugs (NSAID), an increased bowel wall permeability is seen. This can also be induced in healthy individuals and control patients by the administration of NSAID [49,102,104]. On the other hand, neither macroscopic nor microscopic lesions were found during ileocolonoscopy of 20 RA patients using NSAID [47].

Bacterial cell wall fragments do occur in the bowel lumen, can be isolated from faeces and have a molecular weight of more than 2 x 10^5 D [96], which is substantially higher than of those of ^{51}Cr-EDTA and PEG. During bacterial growth, PG fragments with a molecular weight ranging from about 10,000 to 300,000 D are secreted in the culture fluid [98]. Uptake of bacterial cell wall compounds through the bowel wall may take place by mechanisms such as pinocytosis, translocation and by M-cells of Peyer's patches [94]. Experimental evidence obtained by the use of low-molecular weight test molecules may be of limited value when the passage of macromolecular bacterial fragments through the bowel wall is studied. To cite Struthers *et al.*: 'If we are to demonstrate that passage of antigenic material through the intestinal wall is abnormal or increased in RA, molecules which resemble such material will have to be used for the tests' [105]. Regarding molecular weight, the findings of Fagiolo *et al.* are more relevant [106]: they used bovine beta-lactoglobulin (M=35,000) absorption as a permeability marker. In untreated RA patients an increased absorption was found, compared with inactive RA and controls; an increased permeability was observed in controls treated with acetylsalicylic acid.

The experiments reported by Sartor *et al.* [107] give some additional information. They studied the uptake of labeled streptococcal CWF administered in the caecal lumen of rats. A small but detectable amount of CWF was demonstrated 48 hrs later in liver, spleen and mesenteric lymph nodes. The uptake of bacterial CWF increased more than tenfold when the colon wall was injured by the intraluminal injection of dilute acetic acid. Thus, in animal studies the uptake of bacterial cell wall fragments from the bowel lumen has been clearly established.

Pritchard *et al.* [108] could not demonstrate the presence of muramic acid in rheumatoid synovial tissues and synovial fluid cells using a mass spectrometric procedure. Recently, on the other hand, muramic acid was shown to be present in septic

synovial fluid. This muramic acid could not be detected in synovial fluid with a low bacterial count [109]. The authors argued that their method may still be too insensitive to detect relevant amounts of bacterial remnants in synovial fluids.

Serum antibodies to peptidoglycan

Some investigators demonstrated elevated serum antibody levels to PG determinants in RA patients [110,111]. Park *et al.*, on the other hand, did not find an increased level of serum antibodies to a D-Ala-D-Ala peptidoglycan moiety in RA patients [46]. Ebringer *et al.* found increased serum antibodies against *Proteus mirabilis* in a group of RA patients [45,112].

Taken together, it can be concluded from this series of reports that the bowel wall is not necessarily abnormal in RA patients [113]. However, the bowel wall might be more permeate to bacterial compounds, as there is some evidence that the immune system of RA patients is more exposed to bacterial antigens.

1.4 ANIMAL MODELS FOR JOINT INFLAMMATION

It is difficult to study the etiological impact of the human intestinal flora in RA. Fortunately, some animal models for arthritis are available; four of them will be discussed here in more detail:

(a) Antigen-induced arthritis;
(b) Collagen-induced arthritis;
(c) Adjuvant arthritis;
(d) Streptococcal cell wall-induced arthritis.

1.4.1 Antigen-induced arthritis

The model of antigen-induced arthritis has been developed by Dumonde *et al.* [114]. In this model, animals are subcutaneously (s.c.) immunized with an antigen (e.g. bovine albumin) and boosted. When albumin is subsequently administered locally in a joint, an arthritis develops which subsides within several days. When the charge of the antigen is modified by methylation or amidation to increase its persistence in the joints, the joint inflammation becomes more chronic [115]. Antigen specific T-lymphocytes play a major role in the development of the arthritis: a joint inflammation can be established in naive mice when antigen specific T-lymphocytes are locally injected in the joint together with the antigen [116]. Even in nude mice an arthritis can be elicited by local injection of specific T-lymphocytes and antigen [116]. When the animal is challenged orally or intravenously (i.v.) after the waning of the acute arthritis a flare-up of the joint inflammation can be achieved [117-119].

This animal model teaches us that: (a) to elicit a joint inflammation, the antigen has to be locally present; (b) antigen-specific T-lymphocytes play a major role in the pathogenesis of the disease; and (c) repeated exposure to the antigen leads to a flare-up of the joint inflammation. Studies in this antigen-induced arthritis model gave us valuable insight in the mechanisms and pathogenesis of arthritis, but regarding the etiology of rheumatoid arthritis, its value is limited because of the artificial antigen.

1.4.2 Collagen-induced arthritis

An arthritis model with a more relevant antigen has been developed by Trentham *et al.* [120]. In this model, animals (mice, rats, rabbits, monkeys) are s.c. immunized with homologous or heterologous collagen type II in complete or incomplete Freund adjuvant. After 10-16 days these animals develop a self-limiting polyarthritis of paw joints which lasts for 4-8 weeks and may lead to joint ankylosis [120-122]. Early lesions are confined to the joint synovium. Erosions were rarely seen; rheumatoid factor could not be demonstrated. Both humoral and cellular mechanisms are involved in the development of the joint inflammation, as the disease can be transferred to naive recipients by collagen-specific antibodies as well as by the intravenous (i.v.) transfer of specific T-lymphocytes [121]. Collagen-induced arthritis has several similarities with RA, but also many dissimilarities: collagen-induced arthritis lacks a spontaneous nature, is self-limiting without exacerbations and remissions, and the cellular and humoral immunity is directed against collagen type II only [123,124].

In man, immunity to cartilage collagen does not seem to play a major role in the pathogenesis of RA: a minority of RA patients show antibodies to native collagen type II, a phenomenon which may occur secondary to cartilage damage [125,126]. Cellular immunity against collagen has been demonstrated using peripheral blood lymphocytes but is regarded to be of minor importance [126].

1.4.3 Adjuvant-induced arthritis

In this model, first described by Pearson [127,128], rats are immunized s.c. with complete Freund adjuvant (CFA), made of killed *Mycobacterium tuberculosis* ground in mineral oil. After a latent period of about 12 days, a majority of the rats develop a self-limiting polyarthritis of limb joints, which lasts for several weeks and wanes in the majority of the animals, leaving deformed and stiff paws. Reimmunization with CFA induces a flare-up of the arthritis [129,130], a phenomenon ignored by some authors [131-133]. The joint inflammation is the most striking feature of a systemic disease. In this model, cellular immune mechanisms play a key role in the pathogenesis. The disease can be transferred to irradiated naive recipients by lymphocytes isolated from donor animals after CFA immunization [134,135]. Arthropathic T-cell lines have been isolated from CFA-immunized rats by Holoshitz *et al.* [136]. They also isolated a T-cell clone

(A2b) by which the disease can be transferred to irradiated naive recipients [137] and another clone (A2c) which protects recipient rats from adjuvant-induced arthritis [138]. This A2c clone does not cause disease itself [138]. Rats can be vaccinated against adjuvant arthritis by pre-immunizing them with the 65 kD heat shock protein of *M. tuberculosis* [139], or with a synthetic nonapeptide identical to an immunodominant part of the 65 kD peptide [140]. It is supposed that antigenic mimicry between the 65 kD mycobacterium protein and the core protein of joint cartilage accounts for the disease [133,139]. However, the spread of mycobacterial compounds to joint tissues shortly after injection of labeled CFA in the base of the tail has been demonstrated [141], and therefore also the local presence of mycobacterial compounds may play a role in the pathogenesis of adjuvant-induced arthritis.

To induce polyarthritis, the *M. tuberculosis* in the CFA can be replaced by cell walls of other microorganisms, e.g. *Streptococcus pyogenes*, *Nocardia* [142,143] and *Neisseria* [144]. Not all bacteria are able to induce an arthritis when administered in mineral oil [142,143]. Adjuvant arthritis induced by CWF of bacteria from the indigenous human flora has not been reported. Recent (unpublished) studies from our group showed that *Eubacterium aerofaciens* CWF ground in incomplete Freund adjuvant (IFA) induced chronic persistent arthritis after s.c. inoculation in the tail base of Lewis rats.

PG has been found to be the arthropathic cell wall compound [145]. The minimal PG structure with arthropathic properties when administered in IFA is the muramyldipeptide (MDP) N-acetylmuramyl-L-alanyl-D-isoglutamine [146-148]. The arthropathic activity of MDP depends on the mineral oil used and the amino acids coupled to the muramic acid: its stereoisomer N-acetylmuramyl-D-alanyl-D-isoglutamine does not induce arthritis [147].

This animal model has not only been used to study the arthropathic properties of isolated bacterial compounds, but also to investigate the influence of the intestinal flora upon an induced joint inflammation. Pearson *et al.* demonstrated that adjuvant arthritis can be induced in conventional as well as germfree (GF) rats [149]. Kohashi *et al.* found that GF F344 rats were highly susceptible to the induction of adjuvant arthritis, whereas specific pathogen-free (SPF) rats developed less severe arthritis and with a lower incidence [150]. GF rats which were colonized with *E. coli* or *Bacteroides* species showed less severe arthritis than SPF rats, whereas GF rats colonized with *Propionibacterium acnes*, *Bifidobacterium* or *Lactobacillus* species developed a more severe arthritis than GF rats. When GF rats were colonized with a mixture of *E. coli* and *Lactobacillus* species, a mild disease comparable to that in SPF rats was seen [151,152]. It is suggested that colonization by Gram-negative bacteria may suppress the disease via their lipopolysaccharide (LPS), whereas Gram-positive bacteria may enhance the disease, perhaps via their PG [152].

1.4.4 Streptococcal cell wall-induced arthritis

Bacterial cell wall-induced arthritis was originally described by Cromartie *et al.* [87]. As we chose to adopt this model for our studies on the arthropathic properties of the human bowel flora, this model will be discussed in more detail. In this model a single dose of cell walls from *Streptococcus pyogenes* in aqueous solution is given intraperitoneally (i.p.) to rats of a susceptible strain. Within 24-48 hrs, the rats develop acute swelling of paw joints accompanied by general malaise, conjunctivitis and sometimes diarrhoea. The acute arthritis is most severe at about one week after cell wall injection. Subsequently it wanes and, after 2-3 weeks, it relapses as a chronic polyarthritis of the limbs. This chronic joint inflammation waxes and wanes, and may last for several months, leading to joint deformation, stiffness and ankylosis. Axial, knee and smaller limb joints are less frequently involved. The arthritis, especially the chronic phase, is clearly dose-dependent [87].

The immune mechanisms which occur during the acute and chronic phase of the joint inflammation differ profoundly. The acute phase is complement-dependent: treatment with cobra venom factor 3 days before bacterial cell wall administration reduces the severity of the acute joint inflammation, whereas the chronic phase remains unaffected [153]. The acute systemic disease may lead to death within 1-2 days after cell wall injection [87,154]. In *Streptococcus* group B injected rats a severe decrease in platelet numbers and plasma fibrinogen levels has been found [155], although this is insufficient to explain the death of the rats. The humoral response which follows cell wall injection is less well correlated with the severity of joint inflammation [156,157]. Nude (athymic) Lewis rats show an acute paw inflammation after i.p. CWF administration, but do not develop chronic joint disease [88,158]. When nude rats are supplemented with lymphoid cells of euthymic littermates, they develop an acute as well as a chronic arthritis after cell wall injection [158]. Therefore it was concluded that T-lymphocytes are essential for the development of a chronic joint inflammation, whereas the acute phase is T-cell independent [88,158]. Additional support for this hypothesis was given by the finding that cyclosporin A inhibits the chronic phase of the arthritis [159]. A recent report on the transfer of streptococcal cell wall (SCW) arthritis to naive, irradiated rats by SCW-specific T-cell lines isolated from arthritic animals again confirmed the central role of the T-lymphocyte in bacterial cell wall-induced chronic arthritis [160].

There is a great difference in arthritis susceptibility among rat strains and sex: Sprague Dawley and Lewis rats are highly susceptible, whereas Buffalo and F344 rats demonstrate a self-limiting acute arthritis without a chronic phase upon i.p. injection of SCW [161,162]. No differences in tissue distribution of cell walls were found between susceptible and resistant rat strains [163,164]. The differences in susceptibility are probably not major histocompatibility complex (MHC)-related, as Lewis and F344 rats share the same MHC [165]. Female rats appeared to be more sensitive than male rats

[162]. Castration or oestrogen administration increased the susceptibility of male rats to chronic arthritis [166]. It is suggested that SCW-induced suppression of T-cell function in the F344 rat is responsible for its unresponsiveness [167]. Recent reports show that Lewis rats have a central nervous system defect in the biosynthesis of corticotropin-releasing hormone, whereas (arthritis resistant) F344 rats have an intact hypothalamic-pituary-adrenal axis activation upon exposure to bacterial cell walls and IL-1 [168,169]. A recent study in RA patients reported disturbances in circadian cortisol patterns, especially in patients with high inflammatory activity [170].

Mice do not develop chronic joint inflammation after i.p., i.v. or intra-articular (i.a.) injection of SCW [171], but show a pancarditis after a single i.p. injection of SCW [172].

Chronicity of the joint inflammation

Chronicity is an important feature of SCW-induced arthritis. Insight into mechanisms which contribute to chronicity in the rat model might help to understand the chronic nature of human RA. Several factors play a role in chronicity:

(a) The molecular size of the injected SCW influence the outcome of cell wall administration: SCW smaller than 5×10^5 D do not induce arthritis whereas fragments up to 5×10^6 D do induce an acute, self-limiting arthritis. Fragments between 5×10^6 D and 500×10^6 D give rise to a joint inflammation with both an acute and a chronic phase. CWF larger than 500×10^6 D produce chronic arthritis without an acute phase [173].

(b) The outcome of the joint inflammation also depends on the injection route: neither the i.v. nor the i.a. route leads to chronic arthritis [73] (table 1), in contrast to the i.p. injection.

(c) It is essential that the peptidoglycan-polysaccharide (PG-PS) complex is intact: the i.p. injection of purified streptococcal PG leads to an acute, self-limiting arthritis without chronicity [87]; the i.p. administration of the purified polysaccharide (PS) component induces transient paw edema [73]. It has been supposed that an increase in synovial vascular permeability induced by PS makes joints more accessible for cell walls, favouring the chronicity of the subsequent arthritis [73]. However, pretreatment of rats before the i.p. administration of SCW to block mast cell degranulation abrogated the acute paw edema but neither prevented SCW deposition in the joints nor the development of chronic arthritis [174]. This argues against the supposed mechanism for the role of cell wall PS in the spread of bacterial antigens.

PG without coupled PS was found to be more sensitive to lysozyme digestion than native PG-PS complexes [175]. It is supposed that the PS attached to PG protects it against *in vivo* degradation by lysozyme, thus contributing to chronicity. SCW that were acetylated to render them more sensitive to lysozyme were found less arthropathic than native SCW after i.p. injection [176].

Table 1. Joint inflammation induced by bacterial cell wall compounds administered via different routes

Injection route	PG-PS*	PG	PS
intraperitoneal	chronic	acute	oedema
intravenous	acute	acute	oedema
intra-articular	acute	acute	oedema

* PG-PS: peptidoglycan-polysaccharide complex.

An i.v. injection of mutanolysin (endo-N-acetylmuraminidase) after an i.p. injection of an arthropathic dose of SCW in rats abrogates the development of chronic joint inflammation [177]. This beneficial effect of mutanolysin possibly is due to a decrease in SCW size, leading to an inability of these digested cell walls to activate complement [178].

The presence of rhamnose in bacterial cell walls is thought to be related to chronicity of the joint inflammation [179,180] but its mechanism is still unclear. Rhamnose might make the bacterial cell wall more resistant to lysozyme digestion, thus contributing to persistence of bacterial cell walls in tissues. According to Lehman *et al.* [180], a bacterial cell wall has to be lysozyme-resistant and to contain rhamnose to induce a chronic arthritis after i.p. injection.

The persistence of bacterial CWF in tissues is assumed to contribute greatly to the chronic nature of the resulting joint inflammation. An i.p. injection of CWF results in a distribution of cell walls in other organs and tissues: cell wall antigens are found in the joints (synovium as well as subchondral bone), liver, spleen and lymph nodes, especially in tissue macrophages [87,88,181,182]. Cell walls are cleared from tissues over the course of weeks to months, but were found to persist for up to three months in joint tissue macrophages after a single i.p. injection [87]. It is supposed that release of CWF from the liver, subchondral bone or other organs leads to flare-ups and so contributes to chronicity of the joint inflammation [88]. Evidence is accumulating that part of the synovial lining cells (the type A cells) are in fact bone marrow-derived macrophages [183]. These lining cells may therefore be responsible for transport of bacterial compounds from bone marrow deposits to the synovium. I.a. injected bacterial cell walls have been shown to persist in macrophages for at least several weeks [184]. The presence of muramic acid in joint tissues of rats, i.p. injected with streptococcal CWF, has been demonstrated by Fox *et al.* by gas-liquid chromatography-mass spectrometry [185]. They did not find muramic acid in naive rat tissue, in contrast to Sen and Karnovsky [101].

The ability of bacterial cell walls to persist in tissues is also illustrated by the

development of liver granulomas in rats i.p. injected with SCW [162]. These granulomas were not found when cell walls of *Lactobacillus casei* were used [186].

Several experimental procedures have been shown to be effective in the reactivation of a joint inflammation. I.v. injection of homologous and heterologous cell wall polymers, e.g. PG or LPS, leads to reactivation of joint inflammation previously induced by the i.a. injection of SCW [187,188]. The repeated i.a. injection of IL-1 in joints previously injured by an i.a. SCW injection results in a series of flares, which leads to a more severe histopathological change than seen after a repeated IL-1 injection in naive joints [189]. Joints which are already damaged are more susceptible to a subsequent joint inflammation [189,190]. Repeated i.v. injections of small doses of SCW lead to a joint inflammation with increasing severity [191]. These experiments made clear that, once a joint inflammation is established, it can easily be reactivated by a cell wall dose too low to induce joint inflammation in a naive animal. A series of reactivations by minimal amounts of bacterial agents may lead to chronicity. In the SCW model, a continuous release of bacterial fragments from tissue depots probably is responsible for chronicity. In RA, bowel-derived bacterial antigens may play such a role in maintenance of chronicity.

The impact of the indigenous bowel flora on joint inflammation has also been demonstrated using the SCW model. As already stated, F344 rats are known to be resistant to induction of chronic arthritis by i.p. injection of SCW [162]. However, GF F344 rats are susceptible to induction of chronic arthritis, and colonization of GF F344 rats by a conventional rat flora leads to an increasing resistance to induction of chronic arthritis [192].

Recently it has been reported that vaccination against SCW-induced arthritis can be achieved by pre-immunization with the 65 kD *Mycobacterium* protein. This vaccination reduces the severity of the acute phase of the joint inflammation and almost completely abrogates the chronic phase of the arthritis [193]. The mechanism of this vaccination probably is the induction of suppression of the SCW-specific T-cell response.

Histological analysis of SCW-induced arthritis

Paw joint sections of rats with an acute SCW-induced arthritis show peri-articular tissue oedema and infiltration of synovial tissue, tendon sheaths and peri-articular tissue, with predominantly polymorphonuclear cells (PMN). Synovial lining cells are swollen and inflammatory cells around synovial vessels can be seen. In the joint cavity, inflammatory cells, mainly PMN, can be found. Cartilage and bone usually are unaffected [87,88,181].

The chronic phase of the arthritis is characterized by a shift in inflammatory cells from PMN to lymphocytes. In the synovium the lymphocytes predominate, but PMN, synoviocytes [194] and macrophages are also present [87,88,181]. The synovial lining shows hyperplasia with an increased MHC class II molecule expression on lining cells [195]. Synovial vascular proliferation is also seen. In the infiltrated synovium, $CD4^+$ T-

lymphocytes dominate, whereas $CD8^+$ T-cells occur at a much lower frequency [88]. The inflamed synovium may enter the joint space and even overlay the cartilage [87,88], a pattern similar to pannus formation in RA. Cartilage and bone are affected in chronic arthritis: marginal cartilage erosions occur adjacent to inflamed synovium. Usually the underlying bone is also damaged [87,88,181]. At multiple locations, a periosteal apposition of new bone tissue can be seen. Destruction of cartilage and underlying bone tissue followed by connective tissue replacement may even lead to fibrous ankylosis of the joint [87,88]. The paw inflammation is not confined to the synovial lining, cartilage and bone; the tendon sheath lining is also affected. PMN and lymphocytes are seen in the tendon sheath, with swelling of the lining cells and PMN in the space between tendon and sheath [87,88,181].

The histopathological pattern seen in inflamed joints of SCW-injected rats has many features in common with RA: synovitis with infiltration by lymphocytes and PMN, and marginal cartilage erosions with bone destruction and apposition of new bone and pannus formation. However, the strong synovial lining cell proliferation found in RA is less expressed in this animal model. Also, the lymphoid follicle-like noduli which are characteristic of RA have not been found in the rat model. Some other effects seen in streptococcal CWF injected rats are also reminiscent of RA. The protracted anaemia seen after i.p. CWF injection (PG or PS alone is not effective) is also observed in RA; it is related to a decreased erythrocyte survival time, a phenomenon also observed in adjuvant arthritis and RA [196]. An unresponsiveness to skin testing with streptococcal CWF and an *in vitro* unresponsiveness of lymph node cells to phytohaemagglutinin stimulation is seen in rats 3-30 days after an arthropathic cell wall dose [197]. The depressed T-cell function found in arthritic rats was associated *in vitro* to the presence of macrophages [198]. This phenomenon has similarities to the anergy found in patients with active RA [199].

Spontaneous arthritis is not only seen in humans but has also been described in rats and mice: Trentham *et al.* reported on a group of 430 naive female Sprague Dawley rats in which two rats with spontaneous arthritis were seen. Joint inflammation with a higher incidence (6%) was observed in total lymphoid irradiated rats. The severity and pattern of joint inflammation were similar to those observed in rats with collagen or adjuvant arthritis [200].

Arthritis is seen not only after *Streptococcus pyogenes* cell wall injection. CWF of *Lactobacillus casei* [186], *Lactobacillus plantarum* [180], Streptococcus group B, C, H, and *Streptococcus mitis* [87], *Streptococcus faecium* [179] and *Peptostreptococcus productus* [179] also have arthropathic properties. In contrast, CWF from *Staphylococcus aureus* [87], *Propionibacterium acnes* and *Methanobacterium formicium* [179] failed to elicit joint inflammation symptoms after a single i.p. injection.

We employed a modification of the SCW model for our studies on the role of anaerobic intestinal bacteria in the etiology of chronic arthritis in man. The two models which use bacterial compounds (adjuvant arthritis and the SCW model) have advantages

as well as disadvantages. In the SCW model the joint inflammation is far more chronic [87,181] than in the adjuvant model [127-130]. This aspect of the adjuvant model disagrees with the chronic nature of RA and the hypothesis that a continuous influx of bacterial antigens from the bowel maintains chronic joint inflammation.

Arthropathic properties of intestinal flora bacteria have, as yet, not been investigated in either model. Regarding the bacterial cell wall model, little information is available. Lehman *et al.* reported on the arthropathic properties of *Lactobacillus* species [164,180], while Stimpson *et al.* chose *S. faecium*, *P. productus*, *P. acnes* and *M. formicium* as representatives of the human indigenous flora [179]. These microorganisms, however, do not represent the group of major bowel residents [52,57-59]. Furthermore, the bacteria to which the bowel mucosa is exposed far outnumber the bacteria which are found on the skin [201], the mucosa of the mouth [202] or the genital tract [203]. The digestive tract is therefore most likely the primary source of exogenous bacterial antigens to which the immune system is exposed.

Taken together, the absence of a specific causative microorganism in RA, the relation between arthritis and bowel inflammation or infection, the inflammatory properties of bacterial cell wall compounds, and the outcome of animal studies, plead for further investigations of the arthritis-inducing properties of bacterial cell compounds originating from the human indigenous intestinal flora.

1.5 INTRODUCTION TO THE EXPERIMENTAL WORK

We started by investigating the arthritis-inducing properties of cell walls from four Gram-positive human anaerobic intestinal flora bacteria which are associated with CD. Three out of the four strains tested were isolated from the faecal flora of a patient with CD. Patients with CD were found to have antibodies to these bacterial strains more often, and with a higher titer than healthy subjects [204]. A serological assay for the diagnosis of CD, using four intestinal anaerobic coccoid rods, is being applied in our laboratory [204,205]. As these intestinal bacteria may not only be relevant for the gastrointestinal symptoms of CD [206,207] but also for the joint disease complicating CD, CWF from these strains were tested in the animal model described above. The procedure and the results are presented in Chapter 2.

Chapter 3 represents a more detailed study of the arthropathic properties of intestinal flora bacteria and describes the arthritis-inducing capacities of nine strains from four representative, Gram-positive, anaerobic bacterial genera from the human intestinal flora. The impact of cell wall rhamnose and cell wall resistance to lysozyme on arthropathic properties is also investigated.

In Chapter 4, several *Eubacterium* species are investigated to clarify the wide range of arthropathic properties found in Chapter 3. A profound variation was observed between the tested *Eubacterium* strains. Using a set of cell walls from the same bacterial

genus, the hypothesis is tested that cell wall rhamnose contributes to chronicity of joint inflammation; also the role of lysozyme sensitivity in the induction of chronic arthritis is discussed.

In Chapter 5 the joint inflammation induced by i.p. *E. aerofaciens* CWF administration is studied at various time intervals and described in more detail. Decalcified joint tissue embedded in paraffin is used to study the histopathological changes. The findings are compared with descriptions of the streptococcal and *Lactobacillus* cell wall-induced arthritis as well as with human RA.

The composition and arthropathic properties of the intestinal flora in RA are investigated in chapter 6. The faecal flora of 10 RA patients is compared with that of 10 healthy subjects with special attention to *Eubacterium* and *Bifidobacterium* species. Cell wall preparations from *E. aerofaciens* strains isolated from RA patients, as well as healthy subjects, are tested in the bacterial cell wall model.

In the final chapter, I shall discuss the experimental findings and relate these to the hypothesis that the intestinal flora plays a role in the induction and maintenance of chronic joint inflammation in man.

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CHAPTER 2

INDUCTION OF CHRONIC ARTHRITIS IN RATS BY CELL WALL FRAGMENTS OF ANAEROBIC COCCOID RODS ISOLATED FROM THE FAECAL FLORA OF PATIENTS WITH CROHN'S DISEASE

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Digestion 1988;39:118-25.

SUMMARY

Crohn's disease (CD) and ulcerative colitis are accompanied by seronegative arthritis in about one fifth of the cases. In the present study, cell wall fragments (CWF) from major residents such as *Eubacterium*, *Coprococcus* and *Peptostreptococcus* species, isolated from the faecal flora of patients with CD, were tested for properties to induce chronic arthritis in Lewis rats. CWF from *Eubacterium contortum* strains Me44 and Me47 were found to induce chronic arthritis; *Peptostreptococcus productus* strain C18 CWF induced acute self-limiting arthritis. *Coprococcus comes* strain Me46 cell walls, on the other hand, were found to be lethal to the majority of rats inoculated, whereas those which survived did not develop acute or chronic arthritis. These results indicate that intraperitoneal (i.p.) injection of a single dose of CWF from bacteria that are major residents of the human anaerobic faecal flora can induce chronic inflammatory joint disease in the rat.

INTRODUCTION

Both CD and ulcerative colitis are often (14-39%) accompanied by inflammation of one or more large peripheral joints or the spine [1]. The arthritis usually parallels the activity of intestinal disease. The pathogenesis is not known but the higher frequency of arthritis in colonic CD as compared to ileal CD [1,2] suggests a role of intestinal bacteria, known to occur in much higher numbers in the colon than in the ileum. Fifteen per cent of patients with intestinal bypass surgery for morbid obesity develop arthritis, supposed to be due to immune complexes formed as a result of bacterial overgrowth of the surgical blind loop [3,4]. Enteritis caused by *Shigella flexneri*, *Salmonella*, *Yersinia enterocolitica* and *Campylobacter* may be complicated by a sterile arthritis, particularly in patients with the HLA-B27 antigen [5], whereas enteritis due to *Shigella sonnei* is not accompanied by arthritis [6], indicating that small differences between bacteria may also affect the chance on arthritis.

The bowel is a reservoir of foreign antigens to which the host is permanently exposed. Bacterial counts in colonic contents exceed 10^{11} per gram of faeces, more than 99% of which are obligate anaerobic bacteria. Previous studies showed that the faecal flora of patients with CD contained more Gram-positive obligate anaerobic coccoid rods than the flora of healthy subjects [7,8]. Sera from CD patients agglutinated 4 strains of coccoid rods, belonging to *E. contortum* (strains Me44 and Me47), *P. productus* (strain C18) and *C. comes* (strain Me46) species, more frequently and stronger than sera from healthy subjects, patients with ulcerative colitis or other diseases [9-11]. These data indicate that the immune system reacts to antigens from obligately anaerobic bacteria from the intestinal lumen.

An etiological role in rheumatoid arthritis of intestinal bacteria has been proposed

by Bennett [12], supposing that peptidoglycan, the major constituent of Gram-positive bacteria, pass the gut-blood barrier and stimulate the immune system. The purpose of the present study was to investigate whether CWF from major residents of the anaerobic faecal flora of patients with CD could induce arthritis in rats.

MATERIALS AND METHODS

Arthritis was induced in rats by i.p. injection of bacterial CWF as described by Cromartie *et al.* [13] for streptococcal cell walls.

Animals

Groups of five rats were injected i.p. with CWF of *Streptococcus pyogenes*, the coccoid anaerobes (see below) or with sterile phosphate-buffered saline (PBS) as a control. The cell wall dose was adjusted to 33 or 50 μg of muramic acid per g of body weight for *S. pyogenes* and to 25 or 50 μg for the coccoid anaerobes. The animals that received the low cell wall dose of *S. pyogenes* were observed during 50 days, the other during 60 days. Diameters of wrists and ankles were measured with a vernier caliper 5 x in weeks 1 and 2, 3 x in weeks 3 and 4, 2 x in weeks 5 and 6, and once in weeks 7-9. Female Lewis rats (Olac Ltd, Bicester, UK), weighing 150-193 g were used.

Bacteria

Three strains of anaerobic coccoid rods with code numbers Me44, Me47 (*E. contortum*) and Me46 (*C. comes*) were isolated from patients with CD as described previously [8]. *P. productus* strain C18 was obtained from Dr W.E.C. Moore (Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, USA). The strains were cultured in Proteose-Pepton broth (20 g Proteose-Peptone (Oxoid), 1 g glucose; 3 g NaCl; 3 g $K_2HPO_4.3H_2O$; 0.5 g KH_2PO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g cysteine-HCl per liter, pH 7.2), or in Schaedler broth (Oxoid). Bacteria were grown in a 20-liter vessel overnight under anaerobic conditions at 37°C after inoculation with a log phase culture. *S. pyogenes* T3 was obtained from the Bacteriological Laboratory, Academic Hospital Rotterdam Dijkzigt, and cultured overnight at 37°C in Todd Hewitt broth (Oxoid).

Preparation of cell wall fragments

CWF were prepared according to Cromartie *et al.* [13]. In short: cells were harvested, washed, disrupted with glass beads in a Braun shaker (Melsungen, FRG) and, subsequently, collected by centrifugation, washed, lyophilized and treated with

ribonuclease and trypsin, washed and lyophilized again. The crude cell walls were resuspended in PBS and sonicated (MSE, Crawley, UK) at maximal power during 75 min. The sonicated cell walls were passed through a 0.45 μm filter (Schleicher and Schüll, FRG) and tested for sterility on sheep blood agar plates. Muramic acid and rhamnose were determined as described by Hadzija [14] and Dische and Shettles [15], respectively.

Statistical analysis

The differences between the sums of all 4 paw diameters of each individual rat on day 0 and on each observation day were calculated; the median difference of rats injected with CWF from the same bacterial strain were, subsequently, calculated for each observation day; these median values for each observation day for the group of rats injected with cell walls from a particular bacterial strain were compared with the median values obtained in rats injected with CWF from another bacterial strain using the Wilcoxon signed rank test.

Histology

After the observation period, rats were sacrificed by cardiac puncture bleeding under ether anaesthesia. Skinned ankle joints were fixed in 1:10 (v/v) diluted buffered formaldehyde 36% solution, decalcified in 5% (v/v) formic acid during 5 days and subsequently embedded in paraffin. Sections were stained with hematoxylin and eosin.

RESULTS

CWF of *S. pyogenes*

Rats, inoculated i.p. with a single dose of CWF from *S. pyogenes* gradually developed a polyarthritis of the paws, the arthritis being more severe when the high dose was used (Table I and Fig. 1). No arthritis was seen in rats inoculated with saline. These data are in line with results of Cromartie *et al.* [13] and confirm the usefulness of the bacterial cell wall model in our hands.

CWF fragments of anaerobic coccoid rods

Varying degrees of arthritis were observed after i.p. inoculation of rats with different species and strains of the anaerobic coccoid rods (Table I and Fig. 1). In general, more severe acute and chronic arthritis was seen with higher cell wall doses. Cell walls from *E. contortum* strain Me44 induced more severe arthritis than those

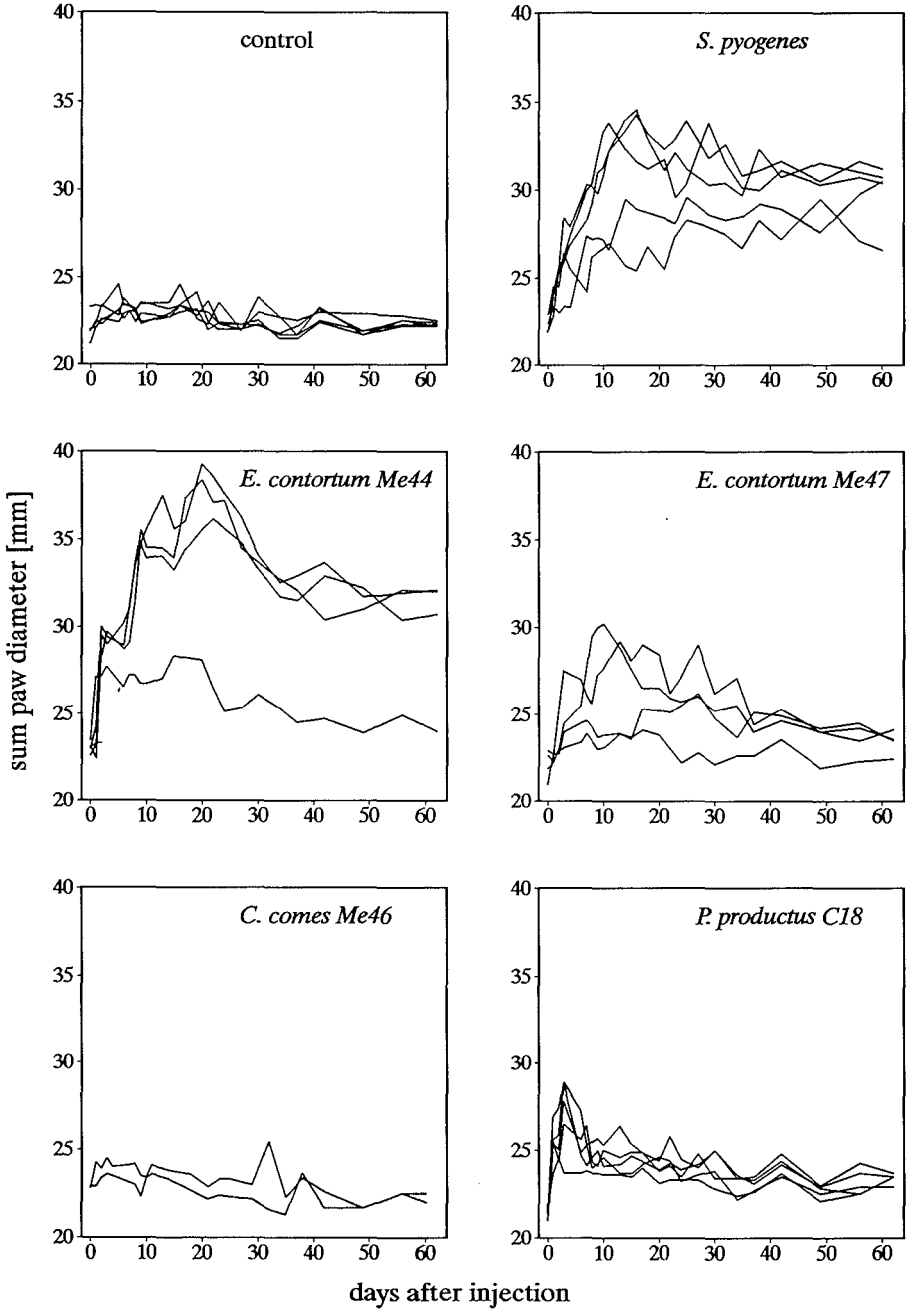

Figure 1. Inflammation induced in rat paws by the i.p. injection of bacterial CWF. Rats were inoculated on day 0 with a cell wall dose of 50 μg muramic acid per g body weight.

Table I. Effect of i.p. injection of CWF from anaerobic Gram-positive coccoid rods from the faecal flora from patients with CD as compared with those from *S. pyogenes*

CWF from bacterium (strain)	Arthritis in surviving rats[1]					
	50 μg muramic acid/g body weight			25 μg muramic acid/g body weight		
	N/S[2]	Acute	Chronic	N/S	Acute	Chronic
S. pyogenes	5/5	+/++	++/+++	5/5	+	+
E. contortum (Me44)	5/4	+++	+++	5/5	++	++
E. contortum (Me47)	5/4	+/++	+	5/5	+	+/-
C. comes (Me46)	5/2	-	-	5/1	-	-
P. productus (C18)	5/5	+/++	-	5/5	+	-
Control	5/5	-	-			

[1]: arthritis in surviving rats, + mild, ++ moderate, +++ severe.
[2]: number of rats injected intraperitoneally/number of surviving rats.

from *S. pyogenes* ($p = 0.0006$), as indicated by the increase of sum paw diameters during the whole observation period. The potency of cell walls to induce chronic arthritis seemed to parallel their rhamnose contents but not their muramic acid contents (Table II).

Table II. Chemical composition of CWF preparations (% of dry weight)

Source of CWF	Muramic acid	Rhamnose
S. pyogenes	11.6	20.4
E. contortum (Me44)	20.1	72.0
E. contortum (Me47)	14.7	40.1
C. comes (Me46)	9.3	0.8
P. productus (C18)	16.4	1.2

Histology

Arthritic joints of rats were histologically examined after the observation period. Hind paw joints of rats inoculated with cell walls of *S. pyogenes* showed extensive infiltration of synovial and periarticular tissues, including tendon sheets, with polymorphonuclear and some mononuclear cells (Fig. 2a). Joint cavities also contained polymorphonuclear granulocytes and mononuclear cells. Synovial lining cells were swollen or destructed, and marginal erosions of cartilage and new bone formation were seen. These results are in line with those from Cromartie *et al.* [13]. Histology of rats inoculated with low dose of *E. contortum* strain Me44 cell walls revealed swollen synovial lining cells and slight cell destruction. Synovial tissue infiltration with mononuclear and some polymorphonuclear cells, marginal cartilage erosions and prominent periostal apposition of new bone were also seen (Fig. 2b).

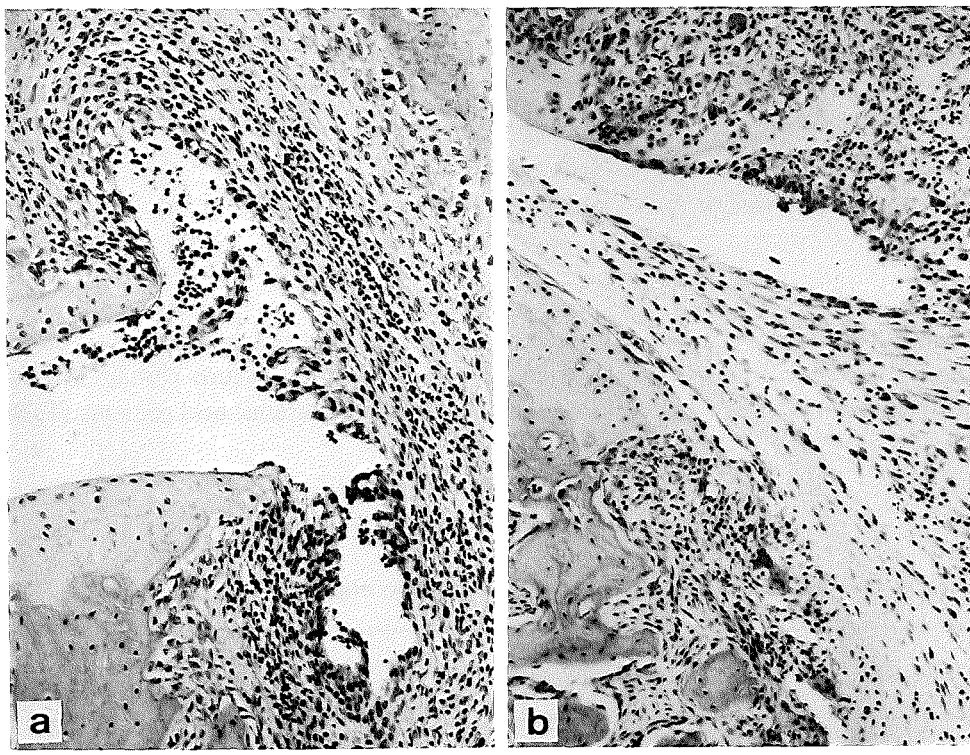

Figure 2. Histological appearance of joint inflammation induced by bacterial CWF: (a) section through a hind paw joint of a rat, sacrificed 43 days after inoculation with low dose of streptococcal CWF. A dense synovial tissue infiltration, synovial lining cell destruction and marginal cartilage erosions are present; magnification: 134 x. (b) section through a hind paw joint of a rat, sacrificed 60 days after i.p. injection of *E. contortum* strain Me44 CWF. Infiltration of synovial tissue and marginal cartilage erosions can be seen; magnification: 108 x.

DISCUSSION

Inability of the host to degrade peptidoglycan-polysaccharide complexes of *S. pyogenes* is supposed to be important for the induction of arthritis or chronic granulomatous reactions in the bowel wall [16-18]. Our results with *P. productus*, inducing self-limiting acute arthritis, are in line with recent results by Stimpson *et al.* [19], who also found that CWF of *Streptococcus faecium*, a facultatively anaerobic bacterium that may occur in small numbers in the human faecal flora, induce chronic arthritis in the rat. The present study shows that single i.p. injection of CWF from *Eubacterium* species, being major residents of the anaerobic faecal flora of both healthy subjects and patients with CD, can produce severe acute and chronic arthritis in the rat.

Rhamnose contents of CWF from *E. contortum* strain Me44 and *P. productus* strain C18 were found to be 72.0 and 1.2%, respectively. The rhamnose contents of *E. contortum* strain Me44 is extremely high but was found on repeated occasions. The rhamnose contents of *P. productus* strain C18, on the other hand, was low when compared with data from Stimpson *et al.* [19]. As this percentage was found repeatedly, we assume that different culture media and techniques as well as different isolation methods may be responsible for this discrepancy. The potency of cell walls to induce chronic arthritis appeared to parallel their rhamnose contents, a phenomenon observed previously by Stimpson *et al.* [19]. The small number of bacterial strains investigated, however, does not allow definite conclusions at present.

Peptidoglycan-polysaccharide complexes have been detected in synovial and periarticular tissues [20]. The polysaccharide portions of these complexes are probably essential for the induction of chronic arthritis [21]. Biochemical differences between polysaccharide chains of the complexes obtained from different bacterial strains may thus be responsible for variations in capacity of individual strains to induce chronic arthritis.

Bacteria of the indigenous human faecal flora are in close contact with the absorbing surfaces of the intestines. Bacterial CWF may be continuously released in the intestinal lumen; if these fragments are absorbed, they could induce arthritis in susceptible hosts. Genetically determined individual variation of the indigenous anaerobic faecal flora [22], in combination with individual variation of host susceptibility [23] may be expected to lead to highly variable expression of arthritis caused by bacterial CWF from the intestinal lumen.

Sartor *et al.* [17] recently showed that, in addition to arthritogenic properties, CWF from *S. pyogenes* could induce granulomatous enterocolitis after injection in the bowel wall. The present study indicates that the arthritogenic properties of *E. contortum* strain Me44, isolated previously from the resident anaerobic faecal flora from patients with CD [7], are stronger than those of *S. pyogenes*. This finding warrants further study on the possible release of peptidoglycan-polysaccharide complexes in the bowel lumen and the circumstances affecting the release and absorption of bacterial CWF *in vivo*. Absorption of antigens of the anaerobic coccoid rods is likely on the basis of the high incidence of

specific antibodies to these bacteria in CD [10, 11]. In line with the results of Sartor *et al.* [17], absorption of peptidoglycan-polysaccharide complexes from *E. contortum* strain Me44 out of the intestinal lumen could be expected to result in granulomatous intestinal inflammatory reaction.

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CHAPTER 3

CELL WALL FRAGMENTS FROM MAJOR RESIDENTS OF THE HUMAN INTESTINAL FLORA INDUCE CHRONIC ARTHRITIS IN RATS

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SUMMARY

To investigate the involvement of human intestinal flora in joint inflammation, cell wall fragments (CWF) of nine anaerobic Gram-positive bacteria of the human faecal flora were prepared and tested for arthropathic properties in the rat. A single intraperitoneal (i.p.) injection of CWF from *Eubacterium aerofaciens* or *Bifidobacterium* species induced persistent chronic arthritis, in contrast to those from *Eubacterium rectale*, *Clostridium* species and *Lactobacillus leichmanii*. The results show that CWF of major residents from the human faecal flora can induce chronic arthritis in the rat and support the hypothesis that the normal human intestinal flora plays a role in the induction of arthritis in man.

INTRODUCTION

The etiology of rheumatoid arthritis (RA) and other forms of sterile arthritis still remains obscure. Bennett [1] postulated that bacterial products or fragments, originating from the bowel contents, may pass the blood-gut barrier, stimulate the immune system and induce RA. Clinical observations show a relationship between joint inflammation and bowel disease, such as arthritis complicating Crohn's disease (CD) and ulcerative colitis [2], reactive arthritis following bowel infection [3] and jejuno-ileo bypass arthritis which may complicate surgical treatment of obesity [4].

The human bowel flora is characterised by the presence of up to 10^{11} obligate anaerobic bacteria per g of faeces [5]. Therefore, the gut associated lymphoid tissue is in close contact with a huge amount of antigens mainly from anaerobic bacteria during a lifelong period. Some of these bacterial antigens might pass the bowel wall, particularly if the permeability of the bowel wall is increased by intestinal inflammation [6] and give rise to inflammation symptoms at distant locations such as arthritis, a possible mechanism occurring in 15% of patients with Crohn's disease [2].

Arthritis induction in rats by peptidoglycan-polysaccharide complexes from cell walls of *Streptococcus pyogenes* has been extensively described by the group of Cromartie *et al.* [7,8]. We adopted this model for our studies on the induction of arthritis by intestinal flora bacteria. In a first report [9], we investigated in the animal model the arthritogenic properties of CWF from four anaerobic Gram-positive intestinal bacteria, isolated from a patient with CD. Serum antibodies to these strains are frequently found in patients with CD [10], indicating that the immune system reacts to these intestinal bacteria. CWF of *Eubacterium contortum* strain Me44 could induce chronic polyarthritis in rats. According to Moore and Holdeman [5], however, this species represents only a minority of the variety of species by which the human intestinal flora is characterised. The limited number of strains tested in our first report does not permit us to draw firm conclusions about the arthropathic properties of intestinal bacteria in general. Other

literature reports about arthritis inducing properties of residential flora bacteria are confined to *Lactobacillus* species [11,12], *Peptostreptococcus productus* [13] and a few minor indigenous flora bacteria [13]. Thus, the hypothesis that the anaerobic intestinal flora is involved in arthritis in man has received limited experimental support in this animal model. We tested CWF of nine species of Gram-positive bacteria, which are major residents of the human intestinal flora, for arthritis-inducing properties.

Lehman *et al.* [11] and Stimpson *et al.* [13] noticed that their CWF inducing chronic arthritis contained rhamnose as a major polysaccharide residue in common. This intriguing observation prompted us to investigate our bacterial CWF preparations for a relationship between rhamnose contents and the induction of chronic arthritis. The potency of bacterial CWF to induce a persistent chronic arthritis has also been related to the *in vivo* and *in vitro* resistance to lysozyme digestion [11,14]. This resistance to lysozyme digestion is essential for a prolonged presence of CWF in tissues and contributes to chronicity of the joint inflammation. The presence of rhamnose in the polysaccharide moiety may protect the bacterial cell wall against digestion *in vivo* by lysozyme. We tested our CWF preparations for *in vitro* sensitivity to lysozyme and compared these results with the CWF preparation's ability to induce arthritis.

MATERIALS AND METHODS

Animals

Female Lewis rats (Olac Ltd, Bicester, UK), weighing 123-221 g were used throughout the whole study. Groups of 5 rats were injected i.p. with an aqueous suspension of CWF [7] from the anaerobic intestinal bacterial strains or with an equal volume of sterile phosphate buffered saline as a control. A cell wall dose of 25 μg muramic acid per g of body weight has been chosen; some CWF were also tested at a dose of 50 μg of muramic acid per g of body weight. The animals were observed for the development of paw inflammation at regular intervals during 60 days; diameters of wrists and ankles were measured with a vernier caliper 5 x in weeks 1 and 2, 3 x in weeks 3 and 4, 2 x in weeks 5 and 6 and once in weeks 7-9.

Bacteria

Eubacterium aerofaciens ATCC (American Type Culture Collection, Rockville, Maryland, USA) 25986, *Eubacterium rectale*, isolated from faeces of a healthy individual, *Clostridium perfringens*, originating from our laboratory strain collection, *Clostridium ramosum* ATCC 25582, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium adolescentis* ATCC 15703 and *Bifidobacterium* strain M, isolated from a healthy individual, were all cultured on proteose-peptone broth (see below) or on Schaedler broth (Oxoid).

Lactobacillus fermentum 84.1253 was cultured on Schaedler broth (Oxoid) with pH adjusted to 6.1 prior to autoclaving; *Lactobacillus leichmanii* 84.609 was cultured under aerobic conditions on Todd Hewitt broth (Oxoid) supplemented with 0.3% (w/v) dextrose. Both *Lactobacillus* strains were gifts from the National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands.

Proteose-peptone broth contains per liter: 20 g proteose-peptone (Oxoid), 1 g glucose, 3 g NaCl, 3 g $K_2HPO_4.3H_2O$, 0.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g cysteine-HCl, pH 7,2. All bacterial strains were cultured overnight at 37°C after inoculation with a log phase culture under strictly anaerobic conditions unless mentioned otherwise. Isolated strains were identified according to the methods of Holdeman *et al.* [15].

Preparation of cell wall fragments

Bacterial CWF were prepared as described by Cromartie *et al.* [7] with minor modifications. Cells were harvested, washed and fragmented with glass beads in a Braun shaker (Melsungen, FGR); cell walls and intact cells were collected by 10.000 g centrifugation and treated again with glass beads if cell fragmentation was shown to be incomplete on microscopic examination of the Gram-stained cell wall suspension. Cell walls were treated with ribonuclease and trypsin, washed and sonicated (MSE, Crawley, UK) for 75 minutes. After sedimentation of debris, the sonicated cell wall suspension was passed through a 0.45 μm millipore filter and tested for sterility.

Chemical analysis of cell wall preparations

Muramic acid and rhamnose contents were determined as described by Hadzija [16] and Dische and Shettles [17], respectively. The total amount of carbohydrate was determined according to Dubois *et al.* [18] with galactose as standard.

Lysozyme digestion of bacterial CWF

CWF suspensions were diluted in 0.1 M sodium acetate buffer pH 5.0 to an optical density of 560 nm (OD_{560}) of about 0.8. Per mg dry weight CWF, 0.1 mg lysozyme (egg white, Sigma) was added, and during incubation at 37°C the OD_{560} was measured at regular intervals [14]. As a control, CWF suspensions were incubated at 37°C without the addition of lysozyme.

Statistical analysis

The mean increase of sum paw diameter from day 1-15 and day 16-60 after cell wall inoculation, compared with the sum paw diameter at the day of inoculation, was

taken as a variable for the severity of the acute and chronic arthritis, respectively. For each rat injected with the low CWF dose, the mean increase of sum paw diameter during the acute and chronic phase of the observation period was calculated; subsequently, the median value of these mean increases of sum paw diameters were calculated for each group of rats. The correlation between the median increase of the sum paw diameter and the rhamnose content of each CWF preparation in the nine groups of rats was evaluated for both the acute and chronic phase using the Spearman rank correlation test.

Histology

After 60 days, rats were sacrificed by cardiac puncture bleeding under ether anaesthesia. Skinned joint specimens were fixed in 1:10 (v/v) diluted buffered 36% formaldehyde solution, decalcified in 5% (v/v) 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 of 9 intestinal flora strains

The results of i.p. injection of rats with bacterial CWF of intestinal flora strains are presented in Table 1. The low dose of *E. aerofaciens* CWF caused acute inflammation of the paws within 1-3 days, with swelling and redness of ankles, wrists, metacarpal and metatarsal regions. This acute inflammation increased, reaching its maximum intensity between days 8-12, then declined but relapsed at the end of the third week as chronic polyarthritis of moderate intensity which was still present at the end of the observation period (Fig. 1). Major joints of ankles and wrists, together with metacarpal and metatarsal joints were affected, but inflammation of digit joints was infrequently observed. At 6 weeks after i.p. injection, inflammation of a knee joint was seen in one out of five rats. During the first few days after CWF injection rats showed malaise, hemorrhagic conjunctivitis, rhinitis and sometimes diarrhoea. When the high CWF dose was given, a far more severe acute and chronic fulminant polyarthritis was observed with an increase in ankle diameter of more than 100% during the chronic phase. Rats inoculated with CWF of *E. rectale* showed minimal general disease during a few days, but failed to develop joint inflammation symptoms.

CWF of both the *Clostridium* strains did not induce acute or chronic joint inflammation after intraperitoneal injection, even when the high cell wall dose was given. *C. perfringens* CWF appeared to be lethal within 24-48 hrs to half of the rats inoculated, whereas *C. ramosum* CWF had no toxic properties (Fig. 1).

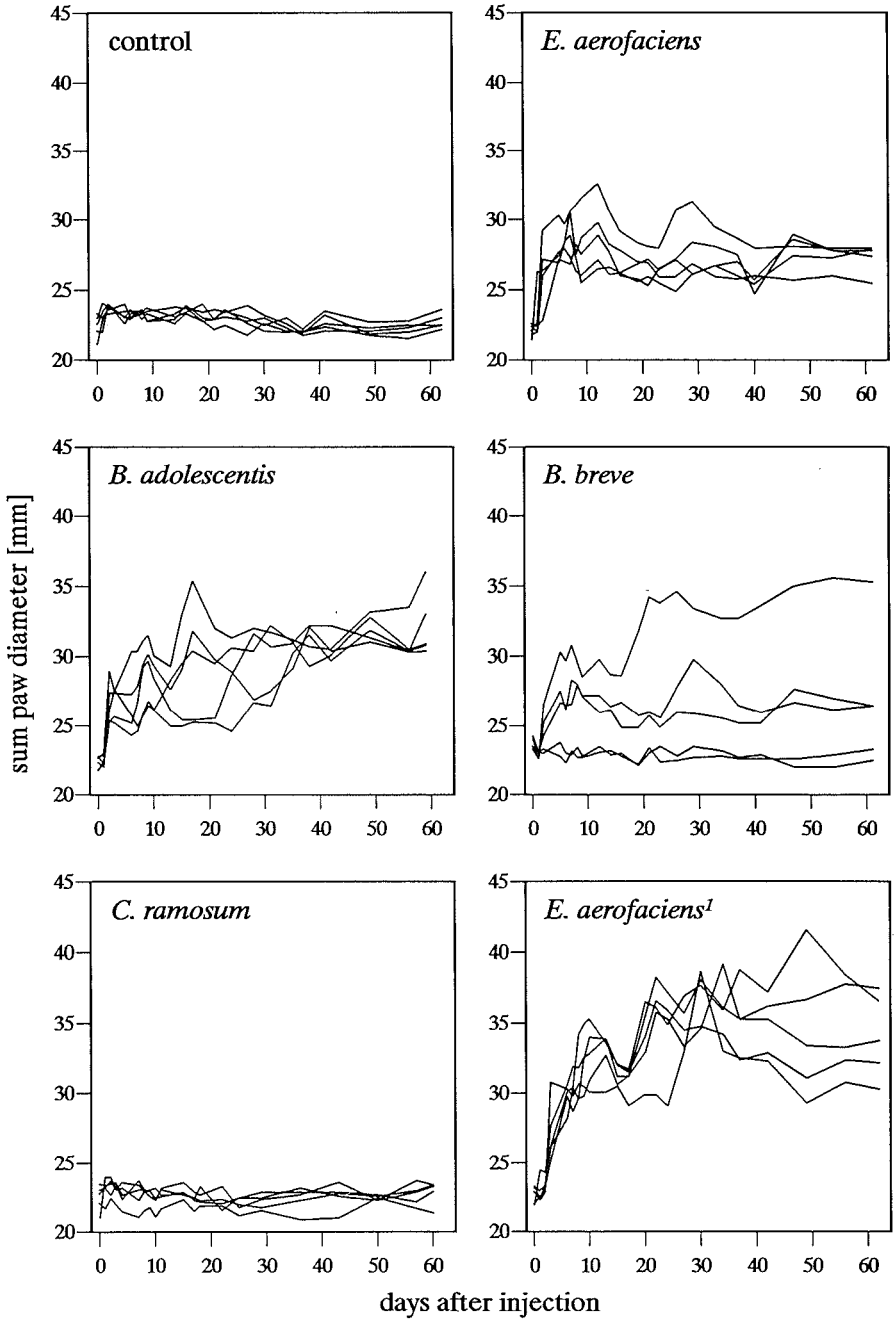

Figure 1. Inflammation induced in rat paws by the i.p. injection of bacterial CWF. Rats were inoculated at day 0 with a CWF dose of 25 μg muramic acid /g body weight; the *E. aerofaciens*[1] group was injected with the 50 μg muramic acid /g body weight dose. Control rats were injected with an equal volume of PBS.

Table 1: Effect of i.p. injection of CWF of anaerobic Gram-positive bacteria from human faecal flora strains

CWF from bacterium (strain)	Arthritis[1]					
	25 μg muramic acid /g body weight			50 μg muramic acid /g body weight		
	N/S[2]	acute	chronic	N/S	acute	chronic
Eubacterium aerofaciens	5/5	++	+/++	5/5	+++	+++
Eubacterium rectale	5/5	-	-	NT[3]		
Clostridium ramosum	5/5	-	-	5/5	-	-
Clostridium perfringens	5/3	-	-	5/2	-	-
Lactobacillus fermentum	5/3	-/+	-	5/3	-	-/+
Lactobacillus leichmanii	5/5	-	-	NT		
Bifidobacterium breve	5/5	+/++	+/++	NT		
Bifidobacterium adolescentis	5/5	+/++	++	NT		
Bifidobacterium strain M	5/5	+	+	NT		
control	5/5	-	-			

[1]: arthritis insurviving rats: - absent, + mild, ++ moderate, +++ severe.
[2]: number of injected rats/ number of surviving rats.
[3]: not tested.

The results obtained after inoculating rats with *L. fermentum* CWF differed: one of 3 rats surviving the low CWF dose injection showed a mild acute arthritis, subsiding during the observation period, whereas 2 of the three rats which survived the high CWF dose injection developed a mild to moderate chronic persisting arthritis without a preceding acute arthritis. Rats inoculated with *L. leichmanii* CWF neither showed signs of paw inflammation nor showed systemic disease after CWF injection.

Bifidobacterium CWF injected rats showed a clinical pattern of paw inflammation comparable to that observed in *E. aerofaciens* CWF inoculated rats. *B. breve* CWF injection resulted within 48 hrs in a moderately acute polyarthritis in three out of five rats followed by a moderate to severe chronic inflammation of paw joints. Two rats did not develop signs of paw inflammation (Fig. 1). CWF of *B. adolescentis* induced a mild to moderate acute paw inflammation within 24-48 hrs after cell wall injection. These rats

gradually developed a moderate chronic polyarthritis, still active 60 days after cell wall inoculation (Fig. 1). One of these 3 rats showed also both knees inflamed near the end of the observation period. Rats which received *Bifidobacterium strain* M CWF developed a mild acute and chronic persisting arthritis. Control rats did not show signs of paw inflammation during the observation period.

Biochemical composition of isolated CWF

The muramic acid contents of the CWF preparations varied from 11 to 20% of dry weight (Table 2). The rhamnose contents of the cell wall preparations showed much more difference from one bacterial strain to another: some CWF contained no more than 1% rhamnose, whereas CWF from other strains contained up to 72% of their dry weight as rhamnose. The low contents of rhamnose, found in some cell wall preparations could not be explained by a low amount of cell wall polysaccharides, as shown by the results of the determination of the total amount of carbohydrates. Table 3 shows the actual dose of cell wall components for each bacterial strain and lists the resulting sum paw diameter increases during the acute and chronic phase of the arthritis. For the 9

Table 2: Chemical composition and lysozyme sensitivity of cell wall preparations

CWF from bacterium (strain)	biochemical composition[1]			lysozyme sensitivity[2]
	muramic acid	rhamnose	total carbohydrate	
Eubacterium aerofaciens	16	31	82	31
Eubacterium rectale	20	1	26	5
Clostridium ramosum	21	72	103	1
Clostridium perfringens	11	7	18	0
Lactobacillus fermentum	15	2	55	61
Lactobacillus leichmanii	15	<1	22	53
Bifidobacterium breve	19	63	102	5
Bifidobacterium adolescentis	11	43	49	15
Bifidobacterium strain M	14	<1	74	0

[1]: results are given as percentages of dry weight.

[2]: expressed as the % decrease of the OD_{560} after 8 hrs incubation with lysozyme, compared with the OD_{560} at time zero.

Table 3: Actual CWF dose and resulting paw diameter increase

Source of CWF	cell wall dose[1]				increase paw diameter[2]	
	muramic acid	dry weight	rhamnose	total carbohydrates	acute	chronic
E. aerofaciens	25	153	47	125	3.91	4.77
E. rectale	25	128	1	33	0.58	0.78
C. ramosum	25	117	84	120	-0.14	-0.24
C. perfringens	25	235	17	42	-0.18	-0.69
L. fermentum	25	172	3	95	0.39	0.36
L. leichmanii	25	171	1	38	0.48	0.26
B. breve	25	127	82	132	2.71	2.49
B. adolescentis	25	224	97	111	4.18	7.48
B. strain M	25	173	2	128	3.49	2.57

[1]: results given as μg per g body weight.
[2]: results given in mm.

strains tested, the correlation between dose of rhamnose, the dry weight of CWF or the total amount of carbohydrates and increase of the sum paw diameters during both phases of the observation period were not statistically significant.

Digestion of bacterial CWF by lysozyme

After incubation with lysozyme, CWF suspensions of some bacterial species showed a decrease of the OD_{560} reaching a plateau at 8 h. The decrease in OD_{560} after 8 h, expressed as percentage of the OD_{560} at time zero, was taken as a variable for lysozyme sensitivity. This *in vitro* sensitivity of the CWF for lysozyme digestion showed great variation (Table 2): CWF from *E. rectale*, both *Clostridium* strains, *B. breve* and *Bifidobacterium* strain M were resistant to lysozymal degradation. CWF of *E. aerofaciens* and *B. adolescentis* were affected by lysozyme, whereas the *Lactobacillus* CWF were found to be rapidly digested by lysozyme. CWF suspensions without lysozyme added did not give a decrease of OD_{560} during incubation at 37°C (results not shown).

Histology

Joint sections

Sections from a hind paw joint of a rat, injected with *E. aerofaciens* CWF showed destruction of joint cartilage and underlying bone with replacement of joint space and adjacent tissues by connective tissue, resulting in fibrous ankylosis (Fig. 2a). Extensive apposition of new bone was seen; no foci with active inflammation were found.

Sections from an arthritic hindpaw of a rat, injected with the *L. fermentum* high cell wall dose also showed extensive new bone formation. Some marginal cartilage erosions were seen and synovial lining appeared to be thickened and sometimes infiltrated, mainly with mononuclear cells. In joint spaces some inflammatory cells, predominantly polymorphonuclear, were found. A few circumscribed periarticular granulomatous inflammatory foci were found, characterised by the presence of histiocytes, mononuclear and polymorphonuclear cells and peripheral fibrosis; multinucleated giant cells and central necrosis were not seen (Fig. 2b).

Infiltration of synovial tissue with both polymorphonuclear and mononuclear cells with some marginal cartilage erosions and pannus formation was seen in hind paw sections of rats, inoculated with *B. breve* CWF (Fig. 2c). Joint spaces and synovial sheaths showed the presence of predominantly polymorphonuclear cells with some macrophages and lymphocytes. Reactive bone formation in the marrow with numerous osteoclasts and prominent periosteal apposition of new bone were also seen. CWF of *B. adolescentis* induced prominent changes in joint structure: joint spaces and cartilage largely disappeared and were replaced by connective tissue. Polymorphonuclear cells were still seen in inflammatory foci. Sections showed periosteal apposition of new bone and an extensive turnover of bone with numerous osteoblasts, osteoclasts and some granulocytes. The bone marrow was also filled with reactive new bone. In contrast, joint sections of rats injected with *Bifidobacterium* strain M CWF showed an almost normal appearance with slight thickening of synovial lining cells and only a few inflammatory cells present in the synovium. No pathological changes of bone or cartilage were seen.

Joints of rats, injected with *E. rectale*, *Clostridium* or *L. leichmanii* CWF, as well as joints of control rats did not show histopathological changes.

Liver sections

All 3 rats which developed chronic arthritis after inoculation with *B. breve* also showed characteristic liver pathology at day 60. The surface of the liver was covered with multiple small white-greyish tumors. Upon histological examination these appeared to be circumscribed, non-caseous granulomas with multinucleated giant cells, histiocytes, lymphocytes and some polymorphonuclear cells (Fig. 2d); some of the granulomas showed central necrosis. The two rats without joint inflammation did not develop macroscopic liver disease. Rats, injected with CWF of *E. aerofaciens*, *E. rectale*, *L. leichmanii*, *B. adolescentis* and *Bifidobacterium* strain M did not show macroscopic liver

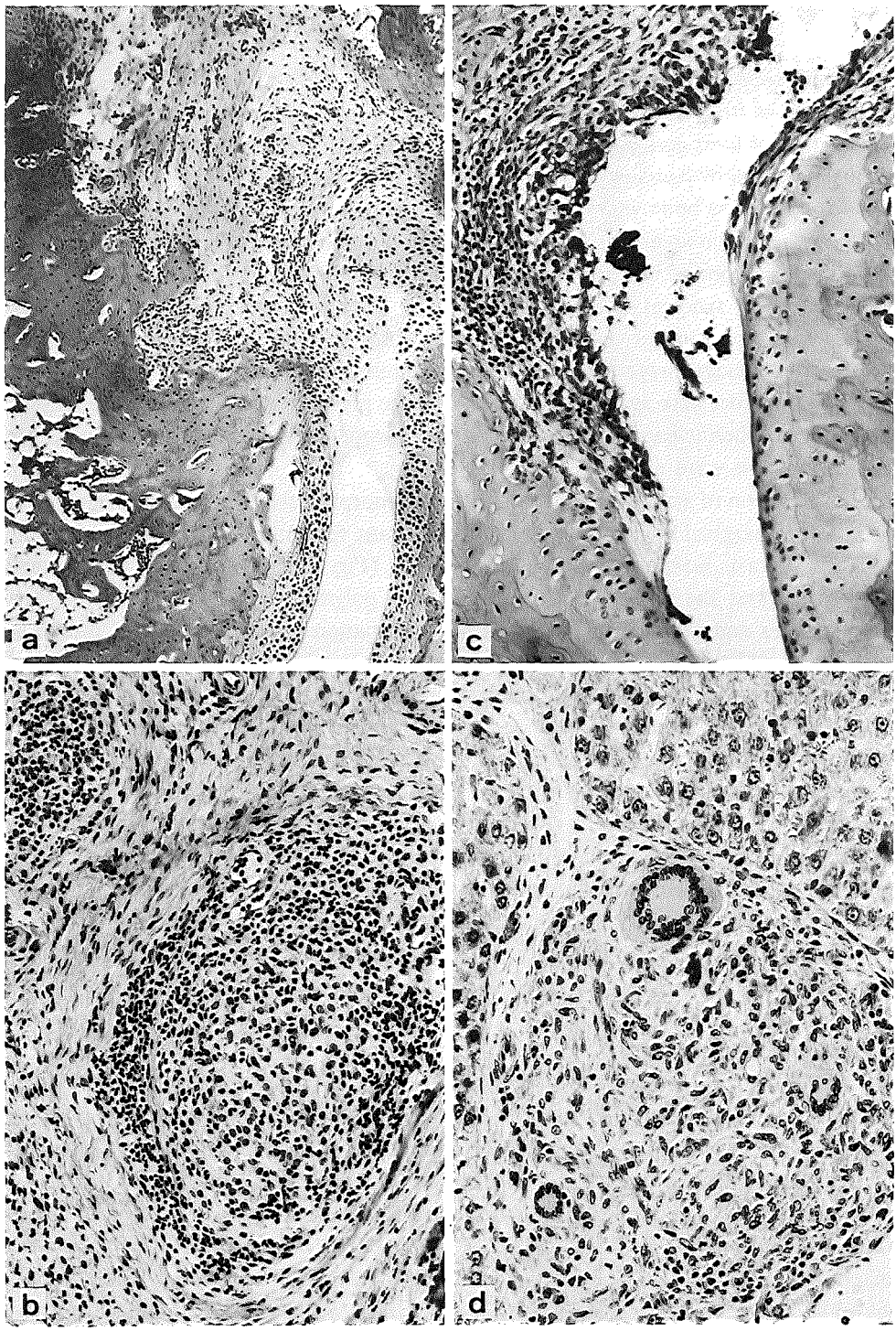
a
c
b
d

Figure 2. Histological appearance of tissue inflammation induced by bacterial CWF, 60 days after i.p. injection:
(a) section through a hind paw of a rat, inoculated with a low CWF dose of *E. aerofaciens*, showing destruction of joint cartilage and underlying bone with replacement by fibrous tissue; magnification: 60 x;
(b) section through a hind paw joint of a rat, injected with a high CWF dose of *L. fermentum*: two circumscribed periarticular granulomatous inflammation foci can be observed; magnification: 152 x;
(c) section through a hind paw joint of a rat, injected with *B. breve* CWF: cartilage erosion and synovial inflammation can be seen; magnification 152 x;
(d) section through the liver of a rat, injected with *B. breve* CWF: surrounded by liver cells, a circumscribed granuloma with several multinucleated giant cells can be seen; magnification: 239 x.

pathology. Rats, inoculated with *L. fermentum* and *Clostridium* CWF had not been observed for liver disease.

DISCUSSION

Our study shows that CWF from *E. aerofaciens* and *Bifidobacterium* strains belonging to major residents of the human indigenous intestinal flora and occurring in numbers of 10^{10} per gram intestinal contents [5,19], induce a severe, longlasting arthritis in Lewis rats after a single i.p. injection. Other major residents such as *E. rectale*, *L. leichmanii* and *Clostridium* species failed to do so. These findings extend previous results showing the arthropathic properties of *E. contortum* isolated from the indigenous flora of a CD patient [9]. As these microorganisms occur in great numbers in the faecal flora of healthy individuals [5,19], these findings emphasize the relevance of the indigenous intestinal flora as a source of exogenous antigens possibly involved in joint inflammation, as recently was remarked by Saag and Bennett [20] and Inman [21].

Stimpson *et al.* [13] also investigated the arthropathic properties of cell wall polymers from normal flora bacteria using the same animal model. They discussed the hypothesis that the normal flora harbours a sufficiently diverse population capable of inducing a wide range of arthropathic responses in the host. They found that, among the limited number of strains tested, only *Streptococcus faecium* induced a persistent chronic arthritis. *S. faecium*, however, is a minor resident occurring in numbers less than 10^8 per gram intestinal contents, a hundredfold lower than the arthropathic *E. aerofaciens* and *Bifidobacterium* strains [5,19] used in this study. So, our results, showing a wide range of arthropathic properties present in several bowel flora bacteria, add substantial experimental evidence to their hypotheses.

Lehman *et al.* [11] and Stimpson *et al.* [13] suggested that induction of chronic arthritis is related to the rhamnose contents of the bacterial cell wall preparation. As peptidoglycan is indispensable for induction of chronic arthritis [22,23] and because rhamnose appeared to be absent in some of our cell wall preparations, we adjusted our cell wall dose on its muramic acid contents instead of the rhamnose contents. This made

it possible to check the suggestion of Lehman and Stimpson, using the variable contents of rhamnose in the nine bacterial cell wall preparations. The data from our experiments grossly follow the suggested trend but, as the correlation found between rhamnose dose and the severity of the arthritis was not significant, we cannot give additional experimental support to their hypothesis. The heterogeneity of the tested bacterial strains, originating from several bacterial genera, may mask the possible relationship between rhamnose contents and chronic arthritis.

Lehman *et al.* [11] also provided evidence that bacterial cell walls which are resistant to lysozyme may induce acute arthritis, but the cell walls must possess a polyrhamnose side chain on the peptidoglycan backbone to induce a chronic arthritis. The CWF of the 9 bacterial strains tested here offer a wide range of lysozyme sensitivities (Table 2), grossly following the formulated hypotheses, but with some intriguing exceptions. In the absence of cell wall rhamnose, the lysozyme resistant *Bifidobacterium* strain M CWF appeared to induce a mild chronic arthritis and the lysozyme resistant *C. ramosum* CWF, possessing rhamnose as major cell wall carbohydrate, failed to induce a chronic arthritis. So, when CWF from several bacterial genera are studied, the relationship between arthropathic properties, lysozyme sensitivity and presence of cell wall rhamnose seems not to be as clearcut as was found by Lehman *et al.* [11] and Stimpson *et al.* [13]. It is probable that other cell wall characteristics also play a part in determining the arthropathic properties of bacterial CWF. As is known from the studies of Kohashi [22,24], peptidoglycan is the arthropathic cell wall component of the bacterial cell wall, with muramyldipeptide as the minimal cell wall structure able to induce joint inflammation in the rat. It is known that minimal differences in peptidoglycan composition and structure have a profound influence upon arthropathic properties of bacterial cell wall compounds. The amino sugar backbone of the peptidoglycan is an almost identical structure for all bacteria, but the oligopeptide side chains show great variation [25]. Unfortunately, little information is available about the detailed composition and structure of the peptidoglycan and carbohydrate part of these anaerobic bacteria. Thus, a further explanation for the presence or absence of arthropathic properties of the tested bacteria would be highly speculative.

Histological analysis of joint inflammation showed patterns which are in line with the findings of Cromartie *et al.* [7] for *S. pyogenes* CWF induced arthritis. The profound changes in bone structure induced by *Bifidobacterium* CWF have not been described previously and underline the potent arthropathic properties of these normal flora bacteria. Liver granulomas have been described after i.p. injection with streptococcal CWF [26], but were not seen in rats, inoculated with *L. casei* CWF [12]. The induction of liver granulomas by *B. breve* CWF suggests that these *Bifidobacterium* CWF and streptococcal CWF share mechanisms in tissue distribution and inflammation response. A local chronic granulomatous inflammation reaction upon local injection of streptococcal CWF in the bowel wall was found by Sartor *et al.* [27]. However, *S. pyogenes* is not a major resident of the human faecal flora, whereas *Bifidobacterium* species occur in

high numbers in the human intestinal flora. Absorption of intestinal antigens is likely to occur on the basis of an increase in permeability of the bowel wall observed in patients with CD [6] reflected by the high incidence of serum antibodies to anaerobic coccoid rods found in patients with CD [10]. The presence of high numbers of granuloma-inducing intestinal bacteria in the bowel lumen might well offer an explanation for the bowel wall granulomas found in CD and for the granulomatous rheumatoid noduli complicating RA.

Clinical investigations show a relationship between infection with Gram-negative bacteria and joint disease: spondylitis ankylopoietica has been related to the presence of *Klebsiella* in the stool [28] and reactive arthritis to a *Salmonella*, *Shigella* or *Yersinia* infection of the bowel [3]. Recent observations demonstrate the presence of *Yersinia* antigens in synovial fluid cells of patients with reactive arthritis [29], and in a rat model the arthropathic properties of peptidoglycan structures from *Neisseria gonorrhoeae* are shown [30], whereas a role for LPS in induction and recurrence of arthritis in the rat model is established [31]. The presence of *Yersinia* antigens in synovial fluid cells together with the arthropathic properties of LPS and gonococcal peptidoglycan indicate the relevance of our rat model for the investigation of arthritis associated with Gram-negative bacteria. Further investigations will be necessary to reveal the basis of the arthropathic diversity of CWF originating from intestinal flora bacteria and to elucidate the impact of these bacterial bowel flora antigens on the etiology of arthritis in man.

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CHAPTER 4

CHRONIC ARTHRITIS INDUCED IN RATS BY CELL WALL FRAGMENTS OF *EUBACTERIUM* SPECIES FROM THE HUMAN INTESTINAL FLORA

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SUMMARY

To investigate arthropathic 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 (i.p.) 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 arthropathic 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 arthropathic properties among different species of the anaerobic genus *Eubacterium* and underline the importance of the anaerobic intestinal flora in the induction of joint inflammation.

INTRODUCTION

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 deposition of poorly degradable bacterial compounds in joint tissues [20]. In susceptible individuals, a distortion of balance between the influx of bacterial material and the capacity of the body to clear this material may lead to accumulation of bacterial compounds in tissues, causing inflammation symptoms such as arthritis.

In susceptible rats, a single i.p. injection of 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 of arthropathic 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, occur in high numbers (up to 10^{10} per g of faeces) in the bowel flora 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 arthropathic

properties may also be present between species of a single bacterial genus, as indicated by the profound difference in arthropathic properties of *E. aerofaciens* and *E. rectale* CWF, the latter failing to give any joint inflammation symptoms upon i.p. inoculation.

Differences in arthropathic properties might be related to the biochemical composition of the bacterial cell wall, especially its rhamnose contents [15,26]; 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 arthropathic 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 have 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 i.p. injection.

MATERIALS AND METHODS

Animals

Female Lewis rats (Harlan Sprague Dawley, Bicester UK), weighing 109-197 g were used throughout the study. Groups of four or five rats were injected i.p. with an aqueous suspension of the *Eubacterium* CWF [5]. A cell wall dose equivalent to 25 μg muramic acid was given; control rats were injected with an equal volume of phosphate buffered saline (PBS). 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 7-9.

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, Maryland, USA; *E. rectale* was isolated from the faecal flora of a healthy individual and was identified according to Holdeman *et al.* [11]. All strains were cultured overnight at 37°C on Schaedler broth (Oxoid) 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 given by *Fox et al.* [8]. Briefly, cells were harvested, washed, and fragmented with glass beads in a Braun shaker (Melsungen, FGR). Cell walls were collected by 10,000 g centrifugation, treated with ribonuclease and trypsin, washed, and sonicated (MSE, Crawley, UK) 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 twice at 100,000 g for 60 min. Both 100,000 g pellets were collected, resuspended in PBS and used for i.p. injection after passage through a 0.45-μm-poresize membrane filter (Schleicher and Schüll, FRG) 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 OD_{560} of about 0.8. Per milligram of dry weight of CWF, 0.1 mg lysozyme (egg white, Sigma Chemical Co.) was added, and during incubation at 37°C the OD_{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 paw diameter from days 1-15 and days 16-60 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 phase 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 phase 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 anaesthesia. Skinned-ankle joint specimens were fixed in 1:10 (v/v) diluted buffered 36% formaldehyde solution, decalcified in 5% (v/v) 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 i.p. and shows the resulting changes in sum paw diameter during the acute and chronic phase of the observation period. In general, the *Eubacterium* species differed greatly in their arthropathic 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 periods 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 the animals were affected to the same extent. Rats inoculated with *E. lentum* CWF showed a late start of the chronic phase of the joint inflammation. After 60 days, the livers of these rats were 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.

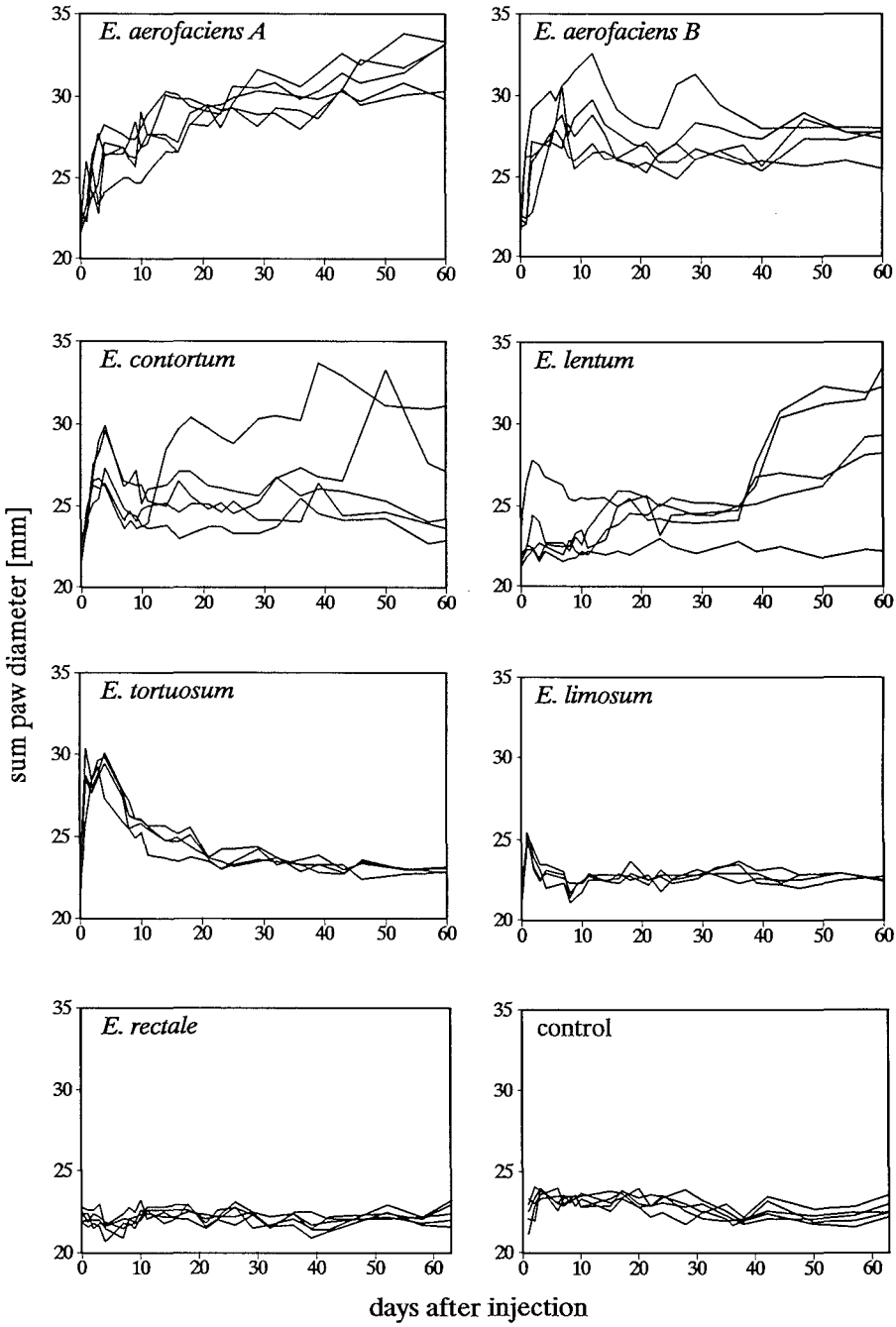

Figure 1. Inflammation induced in rat paws by the i.p. injection of *Eubacterium* CWF; each line represents a single rat. At day 0, rats were injected with a CWF dose equivalent to 25 μg muramic acid per g of body weight. The *E. aerofaciens* A and B CWF were prepared with and without the differential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

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

Source of CWF (n)[a]	Amount of cell wall dose constituent[b]				Increase of sum paw diameter[c] during phase	
	muramic acid	dry weight	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[d]	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] number of rats injected.
[b] results given as μg per g body weight.
[c] results given in mm.
[d] not determined.

Biochemical composition of isolated CWF

The muramic acid contents of the isolated *Eubacterium* CWF varied from 8-20% of 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 for the *Eubacterium* cell wall carbohydrate, the actual amount may be overestimated. 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 diameters during days 15 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 rhamnose dose ($r = 0.18$, $P = 0.71$), nor between the total carbohydrate dose and the severity of the chronic joint inflammation ($r = 0.65$, $P = 0.18$).

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] results given in % of dry weight.
[b] results are given as the % decrease of the OD_{560} after 8 hrs incubation with lysozyme, compared with the OD_{560} at time 0.
[c] not determined.

Lysozyme sensitivity of *Eubacterium* cell wall preparations

The percent decrease in OD_{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 *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 of OD_{560}, except for the *E. aerofaciens* B CWF suspension, which gave a 14% decrease of the OD_{560} after 8 hrs of incubation. No correlation was found when the sensitivity of the *Eubacterium* CWF for 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,

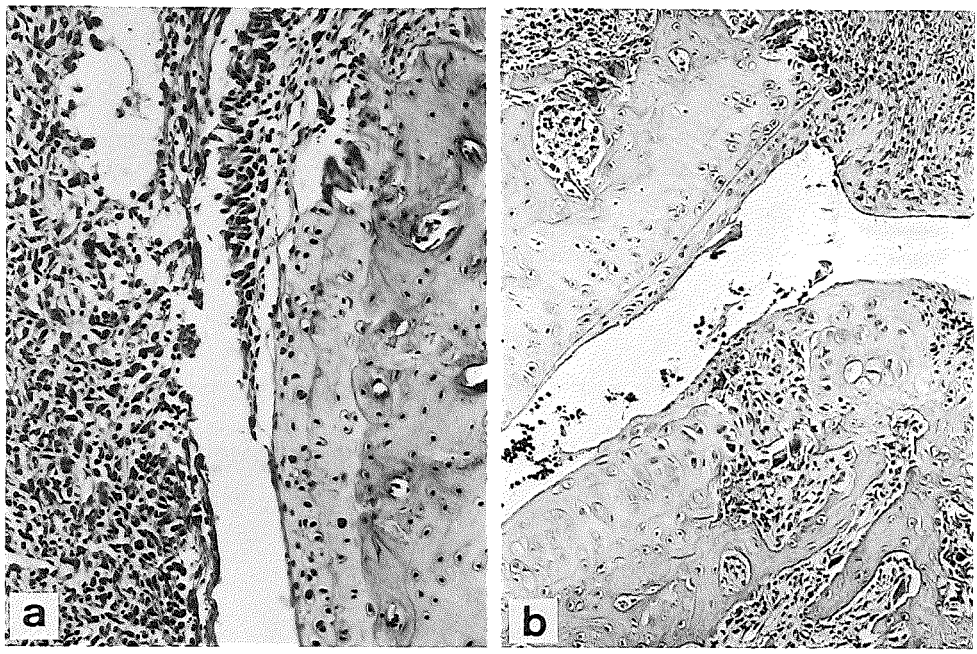

Figure 2. Histological appearance of tissue inflammation induced by *Eubacterium* CWF 60 days after i.p. injection. (a) Section through a hind paw of a rat injected with *E. aerofaciens* CWF. Heavy infiltration of subsynovial tissue with lymphocytes, histiocytes, and some polymorphonuclear cells is markedly present. Also a marginal cartilage erosion with overlying pannus tissue can be seen. Magnification: 95 x. (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: 48 x.

with oedema, infiltration by lymphocytes and vascular proliferation. Heavily infiltrated spots alternated with areas with 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 characterised 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 giants cells were seen in a minority of the granulomas.

Joint sections of *E. tortuosum* CWF injected rats showed 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], which means that *Eubacterium* species occur at numbers of 6×10^9 per gram 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 faecal floras of healthy individuals, i.e., *E. aerofaciens*, *E. contortum*, and *E. lentum* in 29 of 30, 3 of 30 and 8 of 30 healthy individuals, respectively; so, the selected strains are normal flora bacteria.

CWF from the *Eubacterium* species show a spectrum of arthropathic properties, ranging from non-arthropathic (*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. tortuosum*- and *E. limosum*-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 arthropathic properties [22]; the additional centrifugation step 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 harbours bacteria with a wide spectrum of arthropathic 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 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 i.p. injection [27]. Our finding that *E. lentum* CWF are also able to induce these liver granulomas emphasizes the property 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 arthopathic properties. Here we show that, when several bacterial strains from a single genus are examined, a similar variety in arthropathic properties can be seen. Arthropathic and non-arthropathic 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 flora of a patient with Crohn's disease [21].

To clarify the profound differences in arthropathic properties between *Eubacterium* species, we analyzed cell wall characteristics relevant to the bacterial cell wall 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-polysaccharides 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 i.p. 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 arthropathic 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 arthropathic 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 arthropathic properties were degradable by lysozyme, whereas CWF with poor or absent arthritis-inducing capacities were resistant to lysozyme digestion.

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 arthritis; 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 had been demonstrated that minimal changes in the peptidoglycan oligopeptide chains result in profound changes in arthropathic activity [3,13]. Information about these *Eubacterium* cell wall characteristics, which might be also relevant for the CWF 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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CHAPTER 5

HISTOLOGY OF JOINT INFLAMMATION INDUCED IN RATS BY CELL WALL FRAGMENTS OF THE ANAEROBIC INTESTINAL BACTERIUM *EUBACTERIUM AEROFACIENS*

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SUMMARY

To study the arthropathic properties of human intestinal flora bacteria, cell wall fragments of the anaerobic bowel bacterium *Eubacterium aerofaciens* were intraperitoneally injected in arthritis-susceptible Lewis rats. At different stages of the subsequent polyarthritis, rat paw joints were studied for histopathological changes. A persisting synovitis was the hallmark feature, accompanied by marginal erosions of cartilage and bone and a marked periosteal apposition of new bone tissue. These results are discussed in relation to streptococcal cell wall-induced arthritis and compared with histopathological findings in rheumatoid arthritis in man.

INTRODUCTION

In susceptible rats, a single intraperitoneal (i.p.) injection of peptidoglycan-polysaccharide complexes from some Gram-positive microorganisms induce an acute polyarthritis of paw joints followed by a chronic joint inflammation with exacerbations and remissions. This animal model has been described by Cromartie et al [1], using cell walls, cell wall fragments (CWF) and whole cells isolated from *Streptococcus pyogenes* group A. The CWF from *Lactobacillus casei* [2], group D *Streptococcus faecium* [3] as well as some other streptococci [1] also induce a persisting joint inflammation.

This streptococcal cell wall (SCW) model is regarded as a valuable tool in the study of the pathogenesis of acute and chronic joint inflammation [4-6]. The polyarthritis induced by *S. pyogenes* CWF typically shows a biphasic course with a distinct histopathological appearance [1,7,8]. In short: the acute phase is characterized by oedema of articular and periarticular tissues, infiltration by polymorphonuclear (PMN) cells, macrophages and a few lymphocytes, and synovial vasculitis [7,8]. After one week, the acute inflammation clinically [1] and histologically [7] wanes, but is followed by a chronic arthritis, characterized by exacerbations and remissions. In chronically infiltrated joint areas T-helper lymphocytes are the predominant cell type [7], accompanied by macrophages, fibroblast-like synoviocytes [9,10] and a few PMN cells. Tendonitis as well as apposition of new bone and marginal cartilage and bone erosions are seen from two weeks on [7]; the latter may lead to irreparable joint damage [1,7,8].

A microbial etiology of rheumatoid arthritis is strongly suspected [4-6,11,12]. The large bowel is a huge reservoir of microbial antigens to which the host is exposed [13,14]; bacterial compounds from the intestine may pass the bowel wall and be involved in induction or maintenance of chronic joint inflammation [4]. We adopted the SCW model to study the arthropathic properties of CWF from anaerobic Gram-positive bacteria isolated from the human intestinal flora. Reports from our group showed that CWF from *Eubacterium* and *Bifidobacterium* species, which are major residents of the human intestinal flora, can induce chronic joint inflammation in the rat after a single

i.p. injection [15-17]. A further study showed that *E. aerofaciens* was found in high numbers (i.e. $>10^9$ per g faeces) in stool samples of all controls and RA patients studied [18]. In contrast, *S. pyogenes* has infrequently been found in the human intestinal flora [13,14]. CWF from isolated *E. aerofaciens* strains induced chronic arthritis [18]. In these studies, the chronic arthritis was confirmed histologically at day 60 after cell wall administration.

We felt the need to follow the development of the *E. aerofaciens* induced arthritis throughout a prolonged observation period for a number of reasons. For studies on the mechanisms of pathogenesis e.g. by transfer of isolated lymphocytes [19] or vaccination [20] it is important to be well informed about the cellular events *in vivo* to evaluate the effect of intervention. Another reason for our detailed histological study of *E. aerofaciens* CWF induced arthritis is, that *E. aerofaciens* CWF as well as *S. faecium* CWF are clearly more sensitive to lysozyme than CWF of *S. pyogenes* [3,16,17,21]. As chronicity of joint inflammation has been related to antigen persistence in joint tissue [22] which is in turn supposed to be related to in vitro resistance to lysozyme digestion [21,23], the inflammation pattern of *E. aerofaciens* CWF induced arthritis might differ from that induced by *S. pyogenes* cell walls. *S. faecium* CWF are known to induce a less persisting chronic joint inflammation than *S. pyogenes* CWF. Histological investigations, especially when performed during the chronic phase, may throw a light on this item.

In the present paper, we report on the histological analysis of hind paw joint inflammation. At 8 moments during the acute and chronic phase, rats were sacrificed for histological studies. Moreover, we exceeded our previous observation period of 60 days with another 28 days to confirm chronicity of the induced joint inflammation. We also relate our observations to histological descriptions of *S.pyogenes*, *S. faecium* and *L. casei* cell wall induced arthritis.

MATERIALS AND METHODS

Animals

Female Lewis rats (Harlan Sprague Dawley, Bicester, Oxfordshire, United Kingdom) weighing 100-172 g were used during the study. A group of 49 rats were given a single i.p. injection of an aqueous suspension of *E. aerofaciens* CWF. A cell wall dose of 25 μg of muramic acid per g body weight was given; control rats were injected with an equal volume of phosphate buffered saline. The animals were observed for development of paw inflammation at regular intervals during 90 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 7-13.

Bacteria

E. aerofaciens ATCC 25986 was obtained from the American Type Culture Collection, Rockville, Maryland, USA, and was cultured overnight at 37°C on Schaedler broth (Oxoid) under strictly anaerobic conditions after inoculation with a log phase culture. Bacterial CWF were prepared as has been previously described [18] according to Cromartie *et al.* [1] followed by the differential centrifugation procedure of Fox *et al.* [24]. Muramic acid and rhamnose contents of the CWF suspension were determined as has been described by Hadzija [25] and Dische and Shettles [26], respectively. The isolated *E. aerofaciens* CWF contained 15% (dry weight) muramic acid and 29% rhamnose.

Histology

At days 2, 5, 12, 21, 33, 47, 65 and 90 after cell wall injection, a group of 3 or 4 rats was sacrificed for histological study. After cardiac puncture bleeding under ether anaesthesia, left hind paws were skinned and fixed in 1:10 (v/v) diluted buffered formaldehyde 36% solution, decalcified in 5% (v/v) formic acid for five days and embedded in paraffin. Sections of 6 μm were stained with hematoxylin and eosin; some sections were stained with toluidine blue in order to identify mast cells. Of each paw joint about 30 sections, made at 3-4 levels, were evaluated for inflammation symptoms.

RESULTS

Induction of chronic arthritis

The experimental group consisted of 58 rats, including 9 control rats injected with PBS. Of the 49 CWF injected rats, 35 developed a chronic arthritis; 5 rats died within 24-36 h, whereas 9 failed to develop joint inflammation symptoms; the latter were excluded from histological analysis. Depending on the observation time, the 35 positive rats developed acute and chronic joint inflammation, with exacerbations and remissions during the chronic phase. The rats showed malaise, conjunctivitis and diarrhoea during the first days after i.p. cell wall injection. Fig. 1 gives an impression of the induced joint inflammation and shows the arthritis pattern of six rats which were observed for three months: 4 animals developed chronic persisting arthritis, one rat showed an acute self-limiting arthritis whereas another rat failed to develop any joint inflammation symptoms; this rat was one of the 9 excluded from histological analysis. Control rats did not show any clinical signs of paw inflammation.

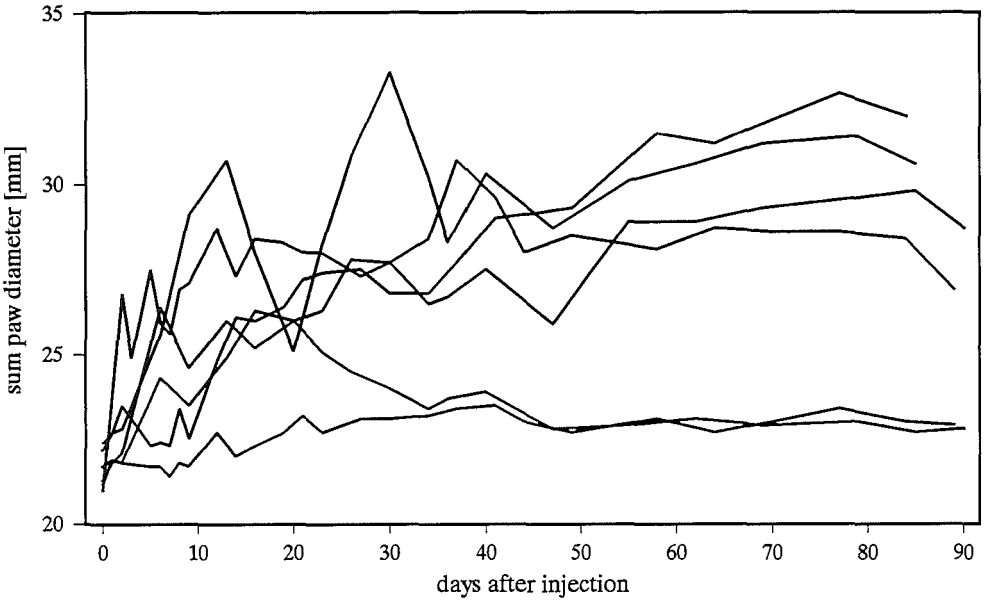


Figure 1. Inflammation of rat paws induced by the i.p. injection of *E. aerofaciens* CWF; each line represents a single rat. At day 0, rats were injected with a CWF dose equivalent to 25 μg muramic acid per g body weight.

Histology

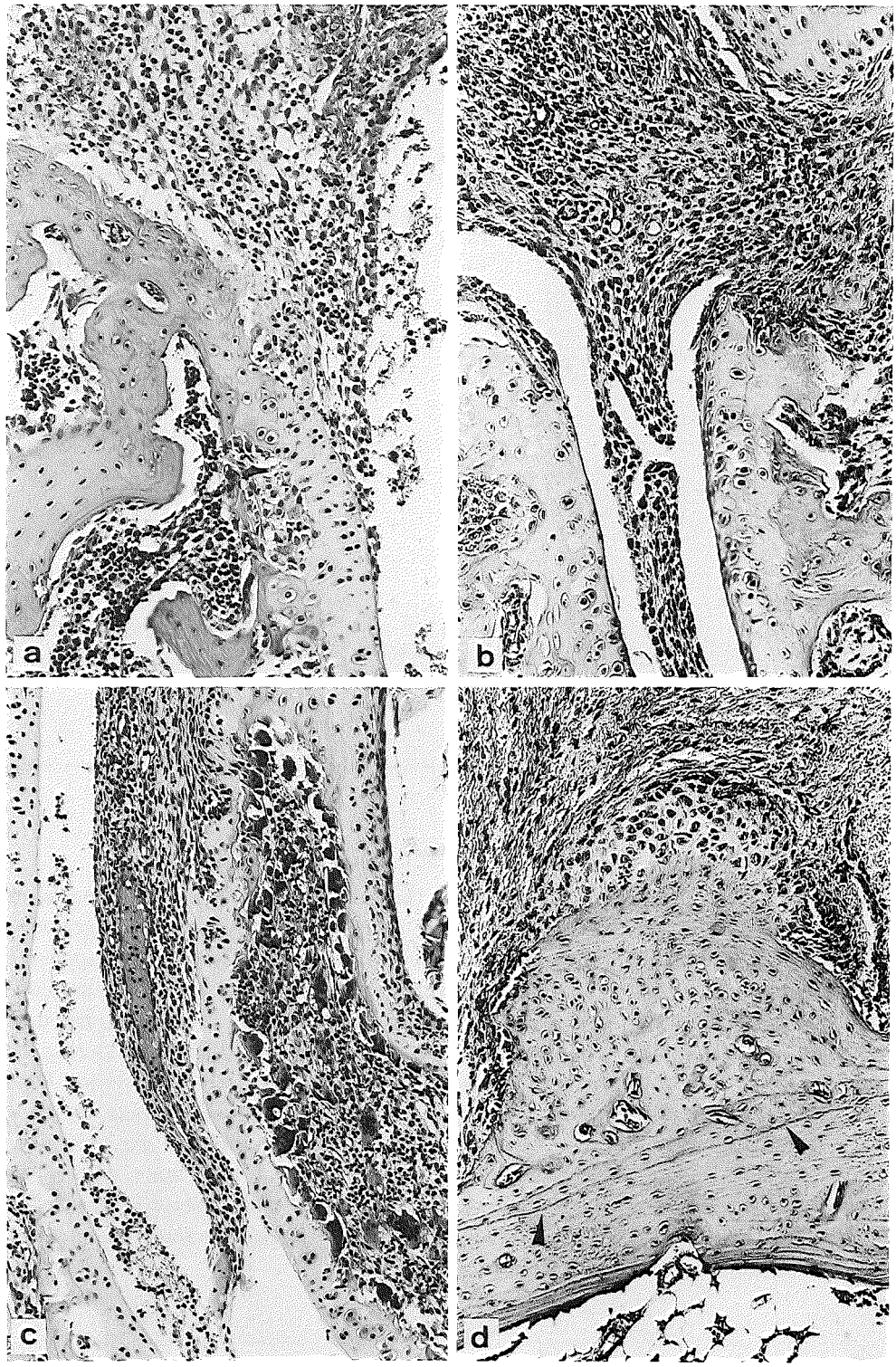
General findings during the development of the joint inflammation are summarized in Table 1. Synovial infiltration was present two days after cell wall injection and was found increased 5 days after cell wall injection; paw sections showed large articular and periarticular infiltrated areas (Fig. 2a). During the acute phase, PMN cells were the major cell type whereas after 2-3 weeks their predominance was taken over by lymphocytes. At the end of the observation period, tissue infiltration waned in some animals: in inflamed synovium and subchondral bone, infiltrated tissue had gradually been replaced by fibrotic connective tissue (Fig. 2f). In contrast, some other rats still showed active synovitis at day 90 (Fig. 2e). Differences in severity of inflammation between areas within a single paw section were found during the whole observation period. Focal proliferation of the synovial lining was a consistent feature of inflamed joints. Synovial fibrosis increased during the chronic phase; tissue oedema disappeared after the first week following CWF injection. Synovial vasculitis was not observed during either the acute or chronic phase. Plasma cells were almost absent during the whole observation period. Mast cells were absent in inflamed synovial tissue, but were clearly

TABLE 1. **Histopathology of joint inflammation induced by *E. aerofaciens* CWF**

	days after cell wall injection							
tissue abnormalities	2	5	12	21	33	47	65	90
synovial infiltration	++*	+++	+++	+++	+++	+/+++	+/++	−/++
PMN cells	++	+++	++	+	+	+	±	±
lymphocytes	+	+	++	++	++	++	+	+
fibrosis	−	−	±	+	+	++	++	++
oedema	++	+	+	±	−	−	−	−
synovial lining hyperplasia	+	++	++	++	++	++	++	+/++
inflammatory cells in joint spaces	+	+	+	±	+	+	+	±
cartilage and bone erosions	−	±	+	+	++	++	++	++
periosteal apposition	−	−	+	+	++	++	++	++
periarticular infiltration	++	+++	++	++	+	+	±	±
tendosynovitis	+	++	++	++	+	+	+	±

*: range; −: absent; +++: maximum severity.

present in loose periarticular tissue. During the chronic phase, some inflamed joints showed pannus tissue extending into the joint cavity (Fig. 2a-c,e). Macrophages were consistently present in infiltrated synovial tissue; fibroblast-like synoviocytes appeared after 2-3 weeks in infiltrated synovium and were especially seen near marginal cartilage and bone erosions. A beginning of marginal cartilage erosion was already seen five days after cell wall injection (Fig. 2a), but was more obvious at day 12 (Fig 2b). During the observation period, erosions became more frequent and spread to underlying bone tissue. Osteoclasts and chondroclasts were seen adjacent to affected cartilage and bone tissue from day 5 on and were also present in infiltrated subchondral bone found near heavily inflamed joints (Fig. 2c). Periosteal apposition of new bone as well as periarticular development of new bone via chondroid tissue was observed from day 12 on (Fig. 2d), and led in some animals to gross paw deformation. In some joint cavities PMN cells with macrophages and fibrin were found.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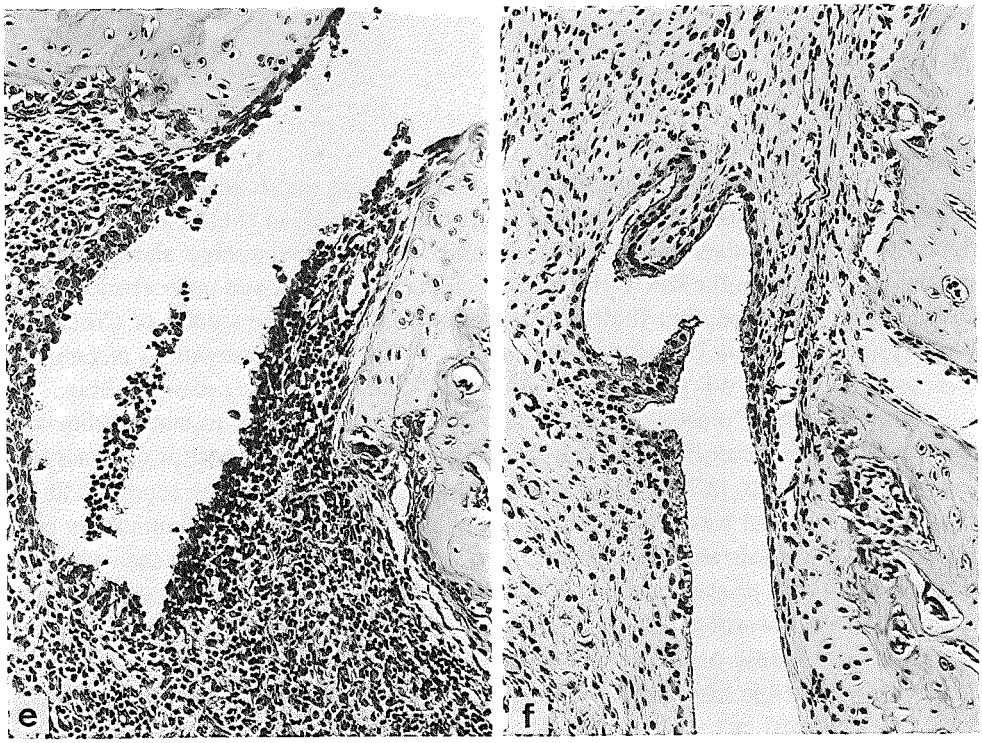

Figure 2. Histological appearance of hind paw inflammation induced by *E. aerofaciens* CWF. (a) Joint section of ankle, made 5 days after i.p. CWF injection. Oedema and infiltration of subsynovial tissue is seen, together with pannus formation and a slight affection of cartilage at the synovium-cartilage transition. The joint space contains some inflammatory cells; magnification: 155 x. (b) Day 12-section: less oedema and more fibrosis of subsynovial tissue is seen when compared to day 5. Marginal erosion of cartilage and underlying bone is apparent; magnification: 124 x. (c) Day 21-section: Extensive pannus formation, overlying the eroded joint cartilage, as well as numerous multinucleated chondroclasts are present. The joint space contains some inflammatory cells; magnification: 155 x. Day 90 sections: (d) Cortical apposition of new bone tissue with a rim of osteoblasts extending into the surrounding connective tissue is seen. The transition zone of cortical bone into new bone tissue is indicated by an arrow; magnification: 155 x. (e) Subsynovial tissue, heavily infiltrated with inflammatory cells, with swollen synovial lining cells are seen, together with marginal erosions of cartilage and bone tissue. Pannus is overlying joint cartilage; magnification: 155 x. (f) Extensive subsynovial fibrosis with few inflammatory cells is observed. Synovial lining cells are flattened; magnification: 124 x.

In periarticular areas, oedema with infiltration by PMN cells and macrophages were observed in the acute phase. At the beginning of the chronic phase, oedema had disappeared but local infiltration was still continuously present; in some paw sections small circumscribed accumulations of lymphocytes were seen. Occasionally, focal accumulations of foamy cells were observed. Synovial or periarticular microabscesses could be observed during the first half of the observation period. Infiltration of tendon

sheaths by PMN cells and some macrophages was seen throughout the whole observation period.

DISCUSSION

The present study confirms our previous observations that *E. aerofaciens* CWF are able to induce an acute and chronic persisting polyarthritis in the Lewis rat [16,17]. Histopathological studies performed at several stages of the disease showed that a continuous synovitis is the hallmark feature, leading to cartilage and bone erosions and, finally, joint destruction. Our findings are grossly in line with the studies of Cromartie *et al.* [1] and Allen *et al.* [7] using streptococcal CWF and with observations of Lehman *et al.* using *L. casei* CWF [2], but they also reveal a few interesting dissimilarities.

The erosions of marginal joint cartilage which we observed to start within two weeks following *E. aerofaciens* cell wall injection were also found in both *S. pyogenes* and *L. casei* injected rats [2,7]: evidence of early marginal erosions was reported to be present at day 10, but was more obvious at day 21. The presence of inflammatory cells in joint spaces was less abundant after *E. aerofaciens* CWF injection than seen after the administration of *S. pyogenes* cell walls. We did not observe vasculitis in synovial tissues, in contrast to observations in the *S. pyogenes* cell wall model [1,18]. Mast cells were found present in loose periarticular tissue but were almost absent in inflamed synovial tissue, a phenomenon also described in antigen-induced arthritis in mice [27]. A study in the streptococcal cell wall model also indicated a limited role for mast cells in the chronic phase of joint inflammation [28].

The biphasic pattern in arthritis induced by *S. pyogenes* CWF has been observed clinically [1] as well as microscopically [7]. However, we did not observe this phenomenon in *E. aerofaciens* CWF induced arthritis. An explanation may be offered by differences in cell wall lysozyme sensitivity. After waning of the acute arthritis a release of CWF from tissue depots as liver, spleen and subchondral bone, together with the influx of T-lymphocytes, may lead to chronicity [7]. Due to an increased lysozyme sensitivity, the release of bacterial CWF from tissue depots may start earlier after *E. aerofaciens* injection than after streptococcal CWF injection, which results in a more continuous arthritis. This reasoning might also be applicable to *S. faecium*: the clinical symptoms of the joint inflammation induced by lysozyme sensitive *S. faecium* CWF were observed to turn smoothly into those of the chronic phase [3]. Unfortunately, the study of Stimpson *et al.* [3] gives too few details to permit the conclusion that also in *S. faecium* CWF induced joint inflammation this biphasic pattern is histologically absent. The arthritis induced by *E. aerofaciens* CWF shares some aspects with that induced by *S. faecium* CWF. Cell walls of both strains induce chronic arthritis without a biphasic pattern, characterized by few exacerbations and remissions, a chronic synovitis with marginal cartilage and bone erosions and formation of new bone tissue. Both *S. faecium* and *E. aerofaciens* CWF contain a reasonable amount of rhamnose (18 and 28% of dry weight, respectively) and are sensitive to *in vitro* digestion by lysozyme [3,17]. We

conclude that our results with *E. aerofaciens* CWF are well in line with observations made in *S. faecium* induced arthritis.

Formation of new bone is an eye-catching feature in both SCW induced arthritis [1,7,8] and *E. aerofaciens* induced arthritis [16,17]. However, in the chronic phase of the *L. casei* model, small bone spurs adjacent to erosions were observed, but no evidence of significant periosteal new bone formation was present [2]; to date it is not understood why these arthropathic and lysozyme resistant [23] cell walls fail to induce marked new bone formation.

When the histopathological findings in the rat model, obtained using *S. pyogenes* [1,7,8] or *E. aerofaciens* CWF, are compared with observations in human RA [29], similarities as well as dissimilarities come to light. Both joint inflammations share synovial infiltration by T-lymphocytes as a hallmark feature. Synovial lining hyperplasia, pannus formation, tendonitis as well as cartilage and bone erosions leading to joint destruction are seen in both rats and humans. The presence of PMN cells in synovial fluid is a feature of both RA and bacterial cell wall arthritis. In contrast to RA, synovial plasma cells as well as follicle-like accumulations of lymphocytes are almost lacking in the rat. The latter are pathognomonic for RA, but are found only in a minority of RA patients. In the chronic phase of the rat model bone apposition is marked, whereas in RA osteoporosis is seen.

Part of the differences between RA and bacterial cell wall arthritis in the rat may be explained by differences in antigen exposure: in RA, an insidious exposure to a low dose of bowel derived bacterial antigens is hypothesized [4-6], whereas in the rat model the disease is elicited by a single, fairly high dose of bacterial cell wall fragments. The initial joint damage caused by this single cell wall dose may have a profound influence on the outcome of the chronic phase of the arthritis in the rat. Unfortunately, no information is available about the effect upon rat joints of a systemic administration of a low dose of bacterial cell walls during a long period. The acute joint inflammation induced by an intra-articular injection of streptococcal CWF can be reactivated by a systemic administration of homologous and heterologous cell wall polymers [29,30]. These observations contribute evidence to the hypothesis that a continuous influx of a low amount of bacterial cell wall does not lead to unresponsiveness to these bacterial antigens but even perpetuates joint inflammation.

E. aerofaciens is found in high numbers in the human large bowel, exceeding 10^9 per g faeces [13,14]; its cell walls have potent arthropathic properties, which share several histopathological features with RA. These findings underline that bowel derived bacterial fragments may well be involved in chronic joint inflammation in man.

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CHAPTER 6

INTESTINAL FLORA OF PATIENTS WITH RHEUMATOID ARTHRITIS. INDUCTION OF CHRONIC ARTHRITIS IN RATS BY CELL WALL FRAGMENTS FROM ISOLATED *EUBACTERIUM AEROFACIENS* STRAINS

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SUMMARY

The composition of the obligate anaerobic intestinal flora of patients with rheumatoid arthritis (RA) differed from that of healthy subjects (HS). Total numbers of aerobes as well as anaerobic coccoid rods were found elevated when compared with HS. *Eubacterium* species were found in high numbers (i.e. > 10^9/g faeces) in all stool samples of both groups; *Bifidobacterium* species were present in 7 (RA) and 8 (HS) out of 10 subjects. From the flora of 2 RA patients and 2 HS *Eubacterium* species were isolated and identified. Cell wall fragments (CWF) from 4 *E. aerofaciens* strains (2 from RA, 2 from HS) were tested for arthritis induction in rats. All 4 strains induced chronic arthritis which was histologically confirmed.

INTRODUCTION

An etiological role for intestinal bacteria in rheumatoid arthritis (RA) has been proposed by Bennett [1] and was recently discussed by Midtvedt [2] and Phillips [3]. In some forms of sterile arthritis, the involvement of bowel bacteria has been established. The role of *Salmonella*, *Shigella*, *Campylobacter* and *Yersinia* in reactive arthritis is clear [4-6]; ankylosing spondylitis has been related to faecal carriage of *Klebsiella* [7] and in Reiter's disease, genito-urinary infection may be complicated by reactive arthritis, especially in HLA-B27 positive individuals [6].

In Crohn's disease (CD) and ulcerative colitis, bowel wall inflammation is complicated in about 20% by arthritis [8]. A microbial etiology in this arthritis is less clear but patients with CD show serum antibodies against anaerobic bowel bacteria [9] and have an abnormal faecal flora [10-12]. CWF from two *Eubacterium contortum* strains, isolated from a CD patient, induce chronic polyarthritis in rats after a single intraperitoneal (i.p.) injection [13]. These findings prompted us to investigate the composition of the intestinal flora of RA patients.

Knowledge about the composition in RA of the most abundant group of intestinal bacteria i.e. the obligate anaerobes is not available. Shinebaum *et al.* [14] compared the faecal flora of RA patients with that of healthy controls but neglected the composition of the anaerobic flora, although anaerobes outnumber aerobes with a factor 100 in a normal flora [15,16]. They demonstrated a higher carriage rate of *Clostridium perfringens* in RA patients but found no other differences. An elevated number of *C. perfringens* in the stool of RA patients has also been described by Olhagen and Månsson [17], but could not be confirmed by other groups [18,19]. A recent report indicated that the jejunal flora may be altered in RA. With the use of jejunal aspirate cultures an abnormal jejunal flora was detected in 5 out of 17 RA patients; all 8 controls were negative [20]. Titres of anti-*Proteus* antibodies were found elevated in the sera of RA patients, in contrast to the level of anti-*Klebsiella* antibodies which did not differ from

that of controls [21].

Several authors have proposed a role for microorganisms in RA [1-3,22,23]. To date, no particular microorganism has been incriminated. The bacterial load from the intestinal flora as a whole may well be a trigger for autoimmune rheumatoid synovitis in predisposed individuals. As the anaerobes constitute more than 99% of the antigenic load of the bowel contents, we focus our investigations on this part of the indigenous flora.

In this paper we deal with two questions: (1) does the anaerobic faecal flora from patients with RA differ from that of healthy individuals? and (2) does it contain species which have arthropathic properties? To test CWF of isolated bacterial species for arthropathic properties, we adopted the animal model described by Cromartie *et al.* [24]. Our previous studies showed that *Eubacterium* and *Bifidobacterium* CWF possess potent arthritis-inducing properties in this animal model [25,26]. In the present paper we compared the faecal flora of 10 RA patients and 10 HS; the faecal flora of two RA patients and two HS was examined for the occurrence of arthritis-inducing *E. aerofaciens* species.

PATIENTS AND METHODS

Ten RA patients were studied, 3 female and 7 male, aged 40-77 years with a median age of 62 years (Table 1). All patients were in-patients and fulfilled the American Rheumatism Association revised criteria [27]. From each subject, 2-3 stool specimens obtained at different days were investigated. During the study, one of the patients used sulphasalazine and another used amoxicillin with clavulanic acid. The control group consisted of 10 healthy subjects (HS), 5 females and 5 males, aged 26-46 years, with a median age of 40 years. Control subjects, who were healthy laboratory volunteers had no joint symptoms or complaints and did not receive antibiotics during the period of stool culture.

Anaerobic culture

Stools were diluted and plated within 2 h after defaecation. Samples of 2 g were suspended in anaerobic broth with a Stomacher Lab Blender (Colworth, London, UK). Three serial 100-fold dilutions were prepared from the suspensions. The anaerobic diluent contained (per litre distilled water): tryptone (Oxoid), 5 g; glucose, 5 g; $K_2HPO_4.3H_2O$, 3 g; KH_2PO_4 0.5 g; NaCl, 5 g; cysteine hydrochloride, 0.5 g and resazurin (BDH), 0.002 g. The pH was adjusted to 7.2 and 100 ml volumes were sterilized for 10 min at 121°C. From the final dilution of the stool sample, 0.2 ml was plated on a non-selective solid medium for anaerobes (Schaedler broth, Oxoid, with 2% agar and 0.0002% resazurin) and incubated at 37°C for 48 h. Anaerobic conditions were verified

Table 1. Composition, arthritis score and medication of the RA patients involved in this study

Patient	age	sex[1]	RI[2]	medication
A	56	M	16	NSAID, penicillamine
B	50	M	14	NSAID, gold salt
C	77	M	2	-
D	76	F	2	NSAID
E	72	M	10	NSAID, penicillamine, sulphasalazine
F	71	F	2	azathioprine, costicosteroids, amoxicillin plus clavulanic acid
G	40	M	10	NSAID
H	65	M	8	NSAID
I	41	F	3	NSAID, gold salt
J	59	M	8	NSAID

[1]M: male, F: female.
[2]RI: Ritchie index.

by using the indicator resazurin which becomes pink at a redox potential above - 120 mV at pH 7.0 [28]. The surface of the solid medium was sufficiently large to enable development of 100 to 150 well-separated colonies. Gram stains of all colonies were made, and the number of isolates was multiplied by the dilution factors to give numbers per gram faeces wetweight. The composition of the flora was determined according to methods used in our previous studies on flora composition [10-12]. In short: the microorganisms were separated in following groups on basis of morphology and Gram characteristics: Gram-negative rods (*Bacteroides* and *Fusobacterium*) and Gram-positive *Eubacterium* or *Bifidobacterium* species, cocci and coccoid rods. More than ninety percent of the cultured organisms could be assigned to one of these groups.

Aerobic counts

Aerobic cultures were performed by plating appropriate dilution samples on sheep blood agar plates; colonies were counted after 24 h of incubation at 37°C.

Identification of *Eubacterium* species

Bacteria which were morphologically assigned as probable *Eubacterium* species were subcultured. Pure cultures were identified according to the system of Holdeman *et al.* [29]. The carbohydrate fermentation capacity was determined in peptone yeast extract medium (PY) with 0.05% L-cysteine-HCl, 0.0002% resazurin and 0.03% agar in sterilized (121°C, 15 min.) test tubes supplemented with 1% (w/v) of the following

filter-sterilized carbohydrates: amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, starch, sucrose, trehalose and xylose. A culture pH < 6.0 after 5 days of incubation was labeled as positive. The glucose fermentation products were determined by gas chromatography from 5-day incubates of the strains in PY broth with 1% glucose at 37°C under anaerobic conditions.

Preparation of bacterial cell wall fragments

The *E. aerofaciens* strains were cultured overnight at 37°C in Schaedler broth (Oxoid) under strictly anaerobic conditions after inoculation with a log phase culture. Bacterial CWF were prepared as described by Cromartie *et al.* [24] followed by the differential centrifugation procedure given by Fox *et al.* [30]. Briefly: cells were harvested, washed and subsequently fragmented with glass beads in a Braun shaker (Melsungen, FGR). Cell walls were collected by 10,000 g centrifugation, treated with ribonuclease and trypsin, washed and sonicated (MSE, Crawley, UK) for 75 minutes. 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 twice at 100,000 g for 60 min. Both 100,000 g pellets were collected, resuspended in phosphate-buffered saline (PBS) and used for i.p. injection after passage through a 0.45 μm millipore filter and subsequent control for sterility.

Chemical analysis of cell wall preparations

Muramic acid and rhamnose contents were determined as described by Hadzija [31] and Dische and Shettles [32], respectively.

Lysozyme digestion of bacterial CWF

CWF suspensions were diluted in 0.1 M sodium acetate buffer pH 5.0 to an OD_{560} of about 0.8. Per mg dry weight CWF 0.1 mg lysozyme (egg white; Sigma Chemical Co., St. Louis, Mo., USA) was added, and during incubation at 37°C the OD_{560} was measured at regular intervals [33]. As a control, CWF suspensions were incubated at 37°C without the addition of lysozyme.

Arthritis induction in rats

Female Lewis rats (Harlan Sprague Dawley, Bicester, UK), weighing 143-195 g were used throughout the whole study. Groups of five rats were injected i.p. with an aqueous suspension of CWF from the *E. aerofaciens* species [24,26]. A cell wall dose equivalent to 24-27 μg of muramic acid per g of body weight was given; control rats were

injected with an equal volume of PBS. The animals were observed for the development of paw inflammation at regular intervals during 60 days; diameters of wrists and ankles at the height of 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 once in weeks 7-9.

The mean increase of sum paw diameter from days 1-15 and days 16-60 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.

Histology

After sixty days, rats were sacrificed by cardiac puncture bleeding under ether anaesthesia. Skinned paw joint specimens were fixed in 1:10 (v/v) diluted buffered 36% formaldehyde solution, decalcified in 5% (v/v) formic acid for five days, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Statistical analysis

The Mann-Whitney U-test was used to calculate the statistical significance of differences in composition of microflora groups of patients with RA and HS.

RESULTS

Composition of the anaerobic flora

The composition of the faecal flora of 10 HS and 10 RA patients is shown in Table 2. Total numbers of anaerobes nor the composition of anaerobic flora did differ significantly between both groups, except the group of coccoid rods: these were found in higher numbers in RA patients than in control subjects ($P = 0.003$). Total numbers of aerobes were also significantly higher in the RA group ($P = 0.0002$). The two patients who used sulphasalazine or amoxicillin with clavulanic acid did not show major differences in flora composition when compared with other RA patients, except a higher number of aerobes. When these two patients were omitted from statistical comparison, the difference between HS ($n=10$) and RA patients ($n=8$) in coccoid rods was still significant ($P = 0.01$), whereas the difference in total aerobes was no longer significant ($P = 0.08$).

In the faecal flora of both RA and HS, *Eubacterium* species were present in all samples and *Bifidobacterium* species in 7 out of 10 and in 8 out of 10 subjects, respectively.

Table 2. Composition of the anaerobic faecal flora of HS and RA patients

Bacterial group	Number of bacteria*		
	HS (n=10)	RA patients (n=10)	P
Total anaerobes	10.62 (10.47-11.37)	10.76 (10.41-11.21)	0.47
Gram negative	10.40 (10.10-10.99)	10.53 (9.97-11.06)	0.29
Gram positive	10.20 (9.94-11.11)	10.13 (10.04-10.50)	0.20
Eubacterium	9.52 (8.93-9.78)	9.70 (8.98-9.96)	0.14
Bifidobacterium	9.14 (<8.00-10.76)	9.16 (<8.72- 9.82)	0.94
Cocci	9.61 (<8.00-9.93)	9.76 (9.39-10.23)	0.09
Coccoid rods	8.46 (<8.00-8.65)	8.98 (<8.56-9.32)	0.003
Other	10.01 (9.74-10.84)	9.75 (9.40-10.32)	0.03
Total aerobes	6.88 (6.00-9.27)	8.40 (<6.82-10.14)	0.0002

* Log_{10} of median value and range per gram faeces (wet wt).

Table 3. Identification of pure cultures of probable *Eubacterium* species selected from the flora of 2 RA patients and 2 HS

Subject	Total number of probable *Eubacterium* species	Identified as:
RA-1	13	9 *Eubacterium aerofaciens* 2 *Eubacterium lentum* 1 *Eubacterium rectale* 1 *Lachnospira multiparus*
RA-2	12	3 *Eubacterium aerofaciens* 3 *Eubacterium rectale* 1 *Eubacterium lentum* 1 *Eubacterium ventriosum* 2 *Peptostreptococcus intermedius* 1 *Peptostreptococcus productus* 1 *Ruminococcus albus*
HS-1	3	3 *Eubacterium aerofaciens*
HS-2	5	2 *Eubacterium aerofaciens* 3 not further identified

Presence of *E. aerofaciens* in flora of RA patients

The faecal flora of two RA patients and two HS was studied in detail. Colonies of bacteria morphologically belonging to the genus *Eubacterium* were subcultured and subsequently identified. Strains able to produce acid from fructose, glucose, lactose, maltose and sucrose and able to produce formic acid, acetic acid, lactic acid and ethanol from glucose were assigned to species *E. aerofaciens*. Otherwise, best fitting species is given. The results are presented in Table 3. We conclude that in the flora of patients with RA as well as from HS *E. aerofaciens* species were present.

Arthritis induction in rats by cell walls from *E. aerofaciens* strains isolated from RA patients and HS

CWF from 4 *E. aerofaciens* strains isolated from the flora of 2 RA patients and 2 HS were tested for arthritis induction. The results are presented in Fig. 1 and Table 4. It is concluded that cell walls from all 4 isolated *E. aerofaciens* strains induced chronic arthritis. CWF from 3 of the 4 *E. aerofaciens* strains appeared toxic after i.p. injection: 1 or 2 rats out of 5 died within 24-36 h. The severity of the paw inflammation differed between the individual rats of a group, a pattern which has also been observed in previous studies. All three rats injected with HS-1 CWF which survived the cell wall injection developed paw arthritis as well as an inflammation of one or both knees during the chronic phase of the observation period. Control rats did not show any sign of paw inflammation.

Table 4. Cell wall dose, sum paw diameter changes after injection, and lysozyme sensitivity of isolated *E. aerofaciens* strains

E. aerofaciens strain	cell wall dose[1]			N/S[2]	sum paw diameter increase[3]		lysozyme sensitivity[4]
	muramic acid	rham-nose	dry weight		acute	chronic	
RA-1	25	1	176	5/4	1.05	2.57	57
RA-2	24	37	135	5/4	2.17	2.17	33
HS-1	27	53	167	5/3	2.74	6.02	43
HS-2	25	7	95	5/5	2.95	2.49	41
control				5/5	0.76	0.44	

[1]expressed in μg per g rat.
[2]number of rats injected/ number of rats survived.
[3]given in mm.
[4]expressed as the % decrease of the OD_{560} after 8 h incubation with lysozyme, compared with the OD_{560} at time zero.

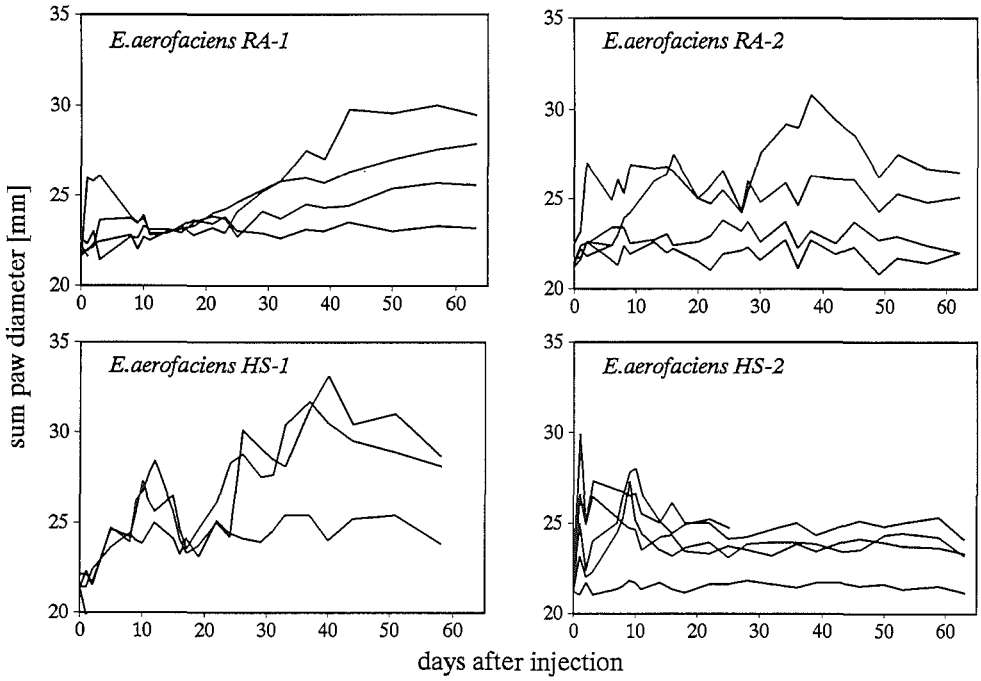

Figure 1. Inflammation induced in rat paws by the i.p. injection of isolated *E. aerofaciens* CWF; each line represents a single rat. At day 0, rats were injected with a CWF dose of 24-27 μg muramic acid per g body weight. The RA and HS strains were isolated from the faecal flora of RA patients and HS, respectively.

We determined the rhamnose contents and the *in vitro* lysozyme sensitivity of the isolated CWF. The findings are shown in Table 4. Two of the strains contained low rhamnose whereas the other strains had fairly high amounts of rhamnose. Regarding the lysozyme sensitivity, the decrease in OD_{560} (Table 4) had reached a plateau after 8 h of incubation with lysozyme; CWF from all four *E. aerofaciens* strains were digested *in vitro* by lysozyme.

Histology

Of all four rat groups, 1-3 representative animals were studied for joint histology. All groups contained animals with one or more affected paws showing a characteristic joint inflammation pattern (Fig. 2). Articular and periarticular infiltration was found; synovial lining cells were swollen and occasionally the synovial lining showed hyperplasia. Synovial tissue was infiltrated predominantly with lymphocytes, synoviocytes [34] and, to a lesser extent, polymorphonuclear cells (PMN). Synovial fibrosis and vascular proliferation was less frequently observed. In some joint cavities, PMN and some

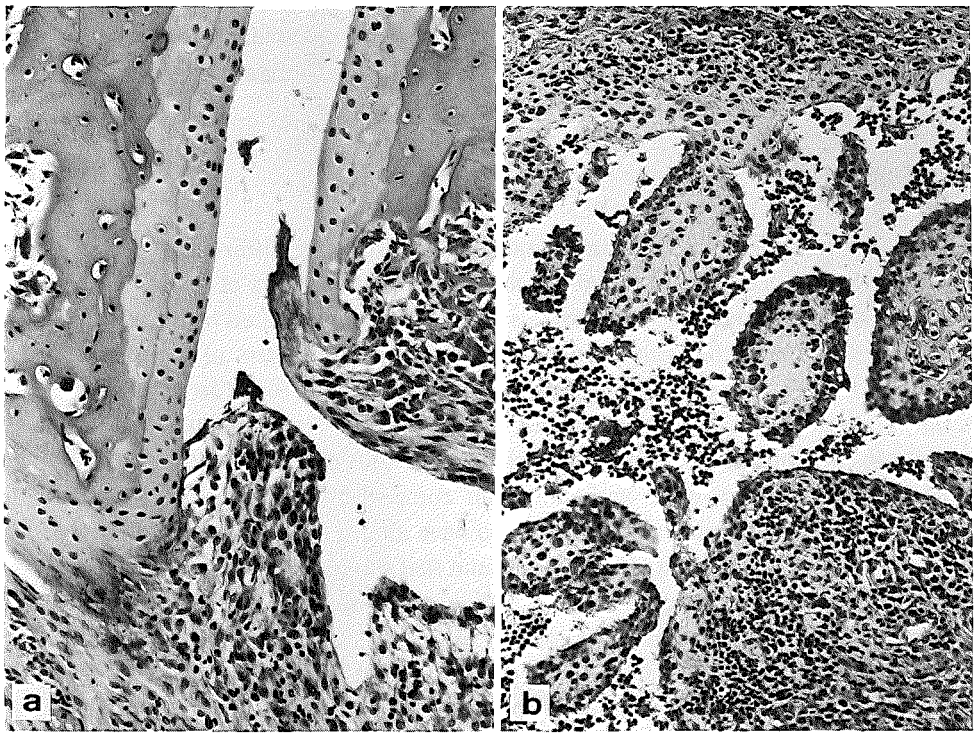

Figure 2. Histological appearance of joint tissue inflammation induced by bacterial CWF, 60 days after i.p. injection: (a) hind paw section of a rat, injected with *E. aerofaciens* HS-2 CWF. Synovial tissue is infiltrated by mononuclear cells; destruction of joint cartilage and underlying bone is seen; magnification: 53 x; (b) section through a front paw of a rat, injected with *E. aerofaciens* RA-2 CWF. Multiple synovial villi with swelling of lining cells are seen; synovial tissue is infiltrated by mononuclear cells. In the joint space, inflammatory cells (predominantly PMN) are found; magnification: 43 x.

mononuclear cells were seen. Adjacent to inflamed synovium, erosions of cartilage and underlying bone were observed, with osteoclasts present. Within a single paw, both affected and unaffected joint tissue was observed. Periosteal apposition of new bone was frequently observed. Occasionally, as seen in a rat injected with *E. aerofaciens* HS-2 CWF, extensive turnover of bone tissue with destruction of synovium and cartilage was markedly present. Tendon sheaths were also affected with swelling of sheath lining cells and infiltration of synovial tissue, predominantly by mononuclear cells. Some paw joints showed one or more periarticular foci consisting of lymphocytes.

Within a single rat, not all paws were affected to the same extent. Grossly, when joint swelling was absent at day 60, no joint pathology was seen or only minor residual abnormalities as slight synovial fibrosis were found. When a major increase in paw

diameter was observed, as in rats inoculated with *E. aerofaciens* HS1 CWF, gross disturbances of joint structure were seen. Joints which were clinically moderately inflamed at day 60 showed active involvement of synovial, cartilage and bone tissue. Paw sections of control rats did not show any sign of joint inflammation.

DISCUSSION

Our investigations of the intestinal flora show some differences between RA patients and HS. Total numbers of aerobes as well as numbers of coccoid rods were found higher in RA patients. The patients and controls investigated by Shinebaum *et al.* were all outpatients; they found no differences in total numbers of aerobes between these groups [14]. The increase in total numbers of aerobes found in our RA patients is not likely to be caused by hospitalization [35] but may be due in part to the use of antibiotics by two patients. This is supported by the shift of P-values seen when the two patients on antibiotics were excluded from comparison. Sulphasalazine as well as amoxicillin plus clavulanic acid are known to have almost no effect on total numbers of the anaerobic intestinal flora [36-38]. We found that the total numbers of anaerobes did not differ between the groups, which is in line with findings of Shinebaum *et al.* [14]. The threefold increase in anaerobic coccoid rods in RA patients is not as impressive as the tenfold increase found in patients with CD [10], but is still intriguing. Serum agglutinins against anaerobic coccoid rods were demonstrated in a minority of RA patients, whereas CD patients showed these serum agglutinins more frequently [9]. In a rat model, the arthropathic properties of CWF from these coccoid rods were demonstrated [13]. The impact of the increased presence of coccoid rods in the faecal flora of RA patients remains unclear yet.

We also showed that in the faecal flora of all investigated RA patients and HS *Eubacterium*-like species occur in numbers of 10^9 per gram faeces. *Bifidobacterium* species, if present, occur in the same numbers but were not detected (i.e. numbers $< 10^8$) in 3 RA patients and 2 HS. Careful isolation and identification of suspect *Eubacterium* species of four flora (2 RA and 2 HS) showed the presence of *E. aerofaciens* in each flora. The presence of *E. aerofaciens* and *Bifidobacterium adolescentis* in high numbers in the intestinal flora was described by Moore and Holdeman [15] and recently by Benno *et al.* [16].

Studies from our laboratory showed that CWF from representatives of the major residents of the obligate human intestinal flora such as *E. aerofaciens* and *B. adolescentis* induced chronic arthritis in Lewis rats [25,26]. In these studies, strains of the American Type Culture Collection (ATCC) were used to avoid doubts about correct identification. We have now demonstrated that CWF of 4 *E. aerofaciens* strains from 4 subjects also induced chronic arthritis after a single i.p. injection in Lewis rats. Thus, *E. aerofaciens* strains isolated and subsequently cultured from individuals with a normal

intestinal flora as well as laboratory strain *E. aerofaciens* ATCC 25986 are arthropathic.

Histological studies showed that the joint inflammation was comparable with our previous findings and in line with studies of Cromartie *et al.* [24] on *Streptococcus pyogenes* CWF. We conclude that in the intestinal flora of RA patients and healthy individuals, *E. aerofaciens* strains are present with cell walls capable of inducing chronic arthritis in susceptible rats. It would be premature to conclude that the intestinal flora of RA patients itself is more arthropathic than the flora of HS; in our opinion, it is more likely that RA patients are more susceptible to the bacterial load originating from the bowel contents.

In two previous papers [25,26] we discussed the hypothesis proposed by two groups using the same animal model [39,40], that the presence of cell wall rhamnose contributes to the arthropathic properties of bacterial cell walls. Our previous studies did not give additional experimental support to this hypothesis, neither do the present findings as given in Table 4. The *E. aerofaciens* strains RA-1 and RA-2 induce chronic arthritis with similar severity, but the rhamnose contents of their cell walls differ greatly (<1% and 27%, respectively). Obviously, the presence of rhamnose in the bacterial cell wall is not a prerequisite for its property to induce chronic joint inflammation, as the arthropathic *E. aerofaciens* RA-1 CWF contain little rhamnose. The present results agree with our previous observations that *E. aerofaciens* CWF are sensitive to *in vitro* lysozyme digestion. This supports our view, that *in vitro* lysozyme sensitivity is necessary for the induction of chronic arthritis [26].

Investigations of the influence of bowel flora bacteria on RA have to include studies on bacterial antigens in synovial tissues or fluids. Bartholomew and Bartholomew [41] demonstrated the presence of polysaccharides immunologically related to *Propionibacterium* species in synovial fluid and synovial leukocytes of RA patients. In RA, experimental evidence for the presence of bacterial compounds, e.g. muramic acid, in synovial tissue is lacking [42]. In reactive arthritis the presence of *Yersinia* and *Salmonella* antigens in synovial fluid cells has been recently demonstrated [4,5]. As stated by Christensson *et al.*, it is premature to conclude that muramic acid is absent in rheumatoid joints since the detection method is too insensitive to demonstrate the small amounts of bacterial antigen that are immunologically relevant [42].

This report describes evidence indicating that the indigenous intestinal flora of RA patients differs from that of healthy individuals by an increased number of anaerobic coccoid rods and total number of aerobes. As the composition of the anaerobic faecal flora is known to be very stable over a long period of time [43], the increase in number of anaerobic coccoid rods in RA might be a characteristic feature and has to be confirmed, e.g. in a group of RA outpatients. From the flora of RA patients and healthy controls *Eubacterium* species have been isolated with arthropathic properties in an animal model. These findings add experimental evidence to the hypothesis that the bacterial load in the intestine may play a role in the etiology of RA.

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CHAPTER 7

GENERAL DISCUSSION

Previous reports have pointed to a possible role for bowel-derived bacterial cell wall compounds in induction or maintenance of chronic arthritis in man [1-3]. Studies directed to this aspect, however, have so far been limited to the aerobic intestinal flora. Since the anaerobic bacteria by far outnumber the aerobic bacteria in the intestinal flora, we have studied the arthropathic properties of cell wall fragments (CWF) from anaerobic intestinal bacteria. This was done in a rat model for chronic arthritis that has many features in common with human RA.

CWF from two *E. contortum* species isolated from the faecal flora of a patient with Crohn's disease (CD) have been shown to induce chronic polyarthritis in Lewis rats after a single intraperitoneal (i.p.) injection (Chapter 2). *P. productus* CWF, on the other hand, induced a self-limiting acute arthritis, whereas *C. comes* CWF failed to induce any joint inflammation in the rats that survived cell wall injection. These findings show that the anaerobic intestinal flora of CD patients harbour arthropathic bacteria; these coccoid rods may play a role in bowel wall inflammation [4,5], but may also be involved in the joint complications associated with CD.

In Chapter 3 we report on the arthropathic properties of a series of representative Gram-positive bacteria from the human anaerobic intestinal flora. We found that CWF of some *Bifidobacterium* species as well as *E. aerofaciens* CWF were able to induce a chronic joint inflammation in the rat, whereas CWF from *E. rectale* and *L. leichmanii* along with two *Clostridium* species gave neither acute nor chronic joint disease. Thus, the potency to induce chronic joint inflammation in the rat is limited to CWF of certain bacterial genera and species. This diversity in arthropathic properties was not only seen between various Gram-positive genera but was also observed within the various species of the genus *Eubacterium* (Chapter 4).

It should be emphasized that these arthropathic anaerobic bacteria are normal inhabitants of the human bowel flora and have been found in numbers sometimes exceeding 10^8-10^9 per g faeces in the large bowel of healthy individuals [6-9]. Our findings (Chapter 6) have confirmed the ubiquitous presence of *Eubacterium* and *Bifidobacterium* species in the faeces of healthy individuals. These important groups of anaerobic Gram-positive bacteria were also found in high numbers in the faecal flora

of RA patients. Absolute numbers of Gram-positive anaerobic coccoid rods as well as absolute numbers of aerobic bacteria were found to be elevated in a group of rheumatoid arthritis (RA) patients, when compared with healthy subjects (HS). It is unknown whether a single species of the coccoid rod group (*Eubacterium*, *Peptostreptococcus*, *Coprococcus* species) or the group as a whole is elevated. The significance of the elevated numbers of anaerobic coccoid rods in RA patients is not clear. As the intestinal flora of healthy individuals consists of about 1.5% anaerobic coccoid rods, the observed threefold increase in coccoid rods is not likely to result in an important increase in intestinal bacterial load. So, the minor differences in intestinal flora composition between RA patients and HS do not plead for a quantitative role for intestinal bacteria in RA, but might indicate a shift in flora composition from a non-arthropathic to an arthropathic combination of bacterium strains. As the resident faecal flora is determined by genetic factors of the host [10], the elevation of coccoid rods in RA might well be an expression of a genetic predisposition to RA. It is intriguing that also in CD these arthropathic anaerobic coccoid rods have been found in elevated numbers [11]. It has been established that *E. aerofaciens* strains isolated from the faeces of both RA patients and healthy individuals are capable of inducing chronic arthritis (Chapter 6). Taken together, it is clear that the intestinal flora of healthy individuals, as well as those of RA patients, harbour a number of anaerobic bacteria with cell walls capable of inducing a wide spectrum of paw inflammation after a single i.p. injection into rats. However, the significance of the changes in bowel flora composition found in RA patients is not clear yet.

In Chapter 5, an extensive histological study of the joint inflammation induced by *E. aerofaciens* CWF was described. We chose the latter bacterium because of its potent arthritis-inducing properties in the rat, and because this Gram-positive anaerobic bacterium is present in high numbers in the intestinal flora of nearly all healthy individuals [6,8]. In some rats, an active joint inflammation was still seen as late as 90 days after cell wall injection. Paw joints inflamed due to i.p. *E. aerofaciens* CWF injection share several histopathological features with inflammation due to *S. pyogenes* [12-14] and *S. faecium* [15] CWF. Furthermore, this joint inflammation has several aspects in common with RA in man [16]. Cell wall properties of *E. aerofaciens* are more like those of *S. faecium* than those of *S. pyogenes*, which is apparent from the absence of a biphasic joint inflammation pattern and the *in vitro* sensitivity to lysozyme.

Our results are an extension of previous studies by other groups in which mainly *Streptococcus* and *Lactobacillus* species were investigated [12,15,17,18]. These studies have contributed valuable information about pathogenetic mechanisms in chronic joint inflammation, but these bacterial species do not represent the human intestinal flora very well [6,8]. The same holds for the minor residents of the bowel flora used by Stimpson *et al.* [15]. Therefore it is questionable whether these studies are relevant with regard to the etiology of RA in man.

The high number of bacterial genera and species tested permitted us to study

bacterial cell wall factors contributing to arthritogenicity. In two studies [12,18] it was hypothesized that the presence of rhamnose contributes to chronicity of bacterial cell wall induced arthritis. Our findings showed several exceptions to this presumed association: for example *C. ramosum* CWF, which are high in rhamnose contents, failed to induce joint inflammation symptoms after i.p. administration (Chapter 3). No positive relationship was found between the rhamnose contents and the arthropathic properties when comparing several *Eubacterium* species (Chapter 4). Other experiments showed that four *E. aerofaciens* cell wall preparations with great differences in rhamnose contents all induced chronic joint inflammation (Chapter 6). So, our findings do not suggest that rhamnose enhances the arthropathic properties. We conclude that this hypothesis, originally based upon the study of some *Streptococcus* and *Lactobacillus* species, is not generally valid.

We also studied the role of *in vitro* cell wall lysozyme sensitivity in the induction of chronic arthritis. It was hypothesized that cell wall resistance to lysozyme is a prerequisite for the induction of chronic arthritis [18,19]. Our observations presented in Chapter 3 do not support this hypothesis. On the contrary, from our results obtained by testing several *Eubacterium* species it became clear that cell wall sensitivity to lysozyme may even favour the chronicity of joint inflammation (Chapter 4). The divergent conclusions of Lehman *et al.* [18] and us may be based upon a different selection of bacteria. Lehman *et al.* based their hypothesis upon the investigation of a small number of bacterial genera i.e. *Streptococcus* and *Lactobacillus*; we found their hypothesis not valid for bacteria from a number of other genera. The question which cell wall factors determine or co-determine the arthropathic properties remains.

Stimpson *et al.* [20] studied the fate of CWF in rat tissues, comparing ^{125}I-labelled cell walls from *S. pyogenes* with those from *S. faecium*. They found that, in contrast to lysozyme resistant *S. pyogenes* CWF, the cell walls of *S. faecium* in liver and spleen were rapidly digested into smaller fragments. However, the amount of CWF in ankle and wrist joints did not differ between these bacterial species. Thus, although both *Streptococcus* species differ profoundly in cell wall lysozyme sensitivity [19], no difference in cell wall persistence in joint tissues was found up to 45 days after cell wall administration. These observations show that lysozyme sensitive *S. faecium* CWF do persist in joint tissues and are able to maintain a chronic joint inflammation after *in vivo* exposure to lysozyme. This observation is in harmony with the data presented in Chapter 4, in which we found a positive correlation between the *in vitro* sensitivity of *Eubacterium* cell walls to lysozyme and their potency to induce a chronic joint inflammation.

In Chapter 4 it is suggested that the composition of the oligopeptide side chains of the peptidoglycan (PG) is an important factor in determining arthropathic properties. Studies of the adjuvant properties [21,22] and arthritis-inducing properties [23,24] of PG fragments have shown that slight variations in side chain composition have a profound influence on their biological activity. Recently, we demonstrated that a series of *Eubacterium* cell walls differed with regard to the amount of oligopeptide side chains

coupled to the muramic acid residues of the PG [25]. This, however, appeared not to be related to their capacity to induce chronic joint inflammation upon i.p. injection.

Our findings that bacterial CWF from several Gram-positive anaerobic bowel bacteria induced persistent arthritis in the rat, give experimental support to the hypothesis that microbial compounds from the bowel contents are involved in chronic joint inflammation in man. Nevertheless, these results have to be interpreted carefully. Our observations have been made in a rat model. A long-lasting exposure to bacterial CWF from the gut, supposed to take place in RA, cannot be realized in the rat. In the rat model, an i.p. bulk dose of bacterial antigens probably results in a continuous release of these antigens from tissues. This might mimic a continuous influx of bacterial antigens from the bowel. However, it is unclear to what extent these conditions are comparable.

Not all rat strains are susceptible to bacterial cell wall-induced arthritis [26], a situation similar to man. As only a minority of individuals exposed to their own bacterial flora antigens develop chronic joint inflammation, a genetic predisposition to RA is theorized. Indeed, RA is known to be related to some extent to the presence of the HLA-DR4 antigen [27].

It should be noted that the bacterial CWF preparations used in our animal model are not identical to the cell wall compounds which are liberated by growing and dividing bacteria [28]. Firstly, the soluble bacterial fragments present in faecal extracts [29] are derived from numerous bacterial species, in contrast to the CWF preparations used in our studies. Secondly, the peptidoglycan fragments liberated as a by-product of growing bacteria have a smaller size (10^4 - 3 x 10^5 D) than CWF obtained by sonication of purified cell walls (up to 5 x 10^8 D) [28,30]. Sonicated CWF with a molecular weight higher than 5 x 10^6 D were arthropathic in the SCW model, whereas PG complexes isolated from bowel contents were found not to be arthropathic after i.p. injection in the rat (unpublished results), probably due to their limited size. This does not exclude arthropathic properties, because, in the adjuvant arthritis model, the induction of arthritis was found less related to cell wall size [23,24] than in the SCW model [30]. Thirdly, as several bacterial and host enzymes are present in the intestine [31,32], the soluble bacterial compounds present in the bowel contents will be subject to digestion by these enzymes. Thus, the bacterial compounds isolated from faeces would be expected to differ not only from CWF of cultured bacteria, but also from bacterial compounds isolated from the proximal part of the colon.

Regarding the etiology of RA, bacterial antigens isolated from faeces are a more relevant antigen than CWF from cultured bacteria. Thus, bacterial cell wall compounds should be isolated from faeces as well as from the contents of the proximal part of the colon, characterized chemically, tested for arthropathic properties and compared to CWF obtained from cultured bacteria, in order to gain more insight into the still obscure relationship between chemical structure and arthrogenicity. Nevertheless, the study of arthropathic properties of mechanically disrupted bacterial cell wall fragments in the rat has given valuable information about the intrinsic inflammatory properties of bacterial

cell wall compounds.

To gather additional experimental support for our hypothesis, it is of great value to investigate whether or not faeces-derived bacterial cell wall structures are present in joint tissues of RA patients. In patients with reactive arthritis, Granfors *et al.* demonstrated the presence of *Yersinia* [33] and *Salmonella* [34] antigens in synovial fluid (SF) cells using an immunochemical method. Pritchard *et al.*, however, were unable to demonstrate muramic acid in SF cells of RA patients [35]. Christensson *et al.*, using the same method (gas-liquid chromatography-mass spectrometry), could not detect muramic acid in some of their septic SF samples [36]. In contrast, with the biochemical assay of Sen and Karnovsky [37] muramic acid was detected in liver, brain and kidney tissue of naive rats. A chemical method has the disadvantage that bacterial compounds can be detected but not localized. Immunohistochemistry using monoclonal antibodies directed to PG structures outweighs this disadvantage, although cross-reactivity between mammalian and bacterial antigens cannot be excluded. The application of both methods to synovial tissues of RA patients and controls should give valuable information about the presence and localisation of bacterial cell wall antigens.

The histopathological studies described in Chapter 5 have given us more insight into the arthritis-inducing properties of *E. aerofaciens* cell walls and the changes of joint structures. We also performed this study to obtain more information about the sequence of cellular changes during the development of joint inflammation. However, not all relevant cell types involved in chronic arthritis can be identified with certainty by using decalcified and hematoxylin and eosin stained paw sections. The use of frozen joint sections stained with monoclonal antibodies directed against surface epitopes of involved cells would optimize the study of the localization and kinetics of T-lymphocytes and macrophages. Should a specific antibody directed to PG structures become available, the combination of these methods to localize both inflammatory cells and bacterial antigens would greatly clarify the relationship between inflammatory cells and peptidoglycan fragments in chronic joint inflammation.

Summarizing, the data presented in the preceding chapters provide strong support for the supposition that CWF isolated from Gram-positive anaerobic bacteria from the human intestinal flora are involved in chronic joint inflammation in man. As the modifying role of cell wall rhamnose and lysozyme sensitivity in chronic arthritis is not supported by our studies, it still remains unclear which properties make bacterial cell walls arthropathic. As bacterial CWF might well differ from those present in bowel contents, intestine-derived bacterial cell wall compounds have to be investigated for chemical composition and arthropathic activity. Further studies should also deal with the occurrence of these bowel-derived PG complexes in rheumatoid joint tissues.

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SUMMARY

Rheumatoid arthritis (RA) is a systemic disease with chronic sterile polyarthritis as a major presenting symptom. It affects 1-2% of the population; its etiology is unknown. For several reasons, an infectious etiology is suspected, but the claims made for several microorganisms have not been convincing. It is hypothesized that the microbial load from the bowel contents may play a role in the induction or maintenance of joint inflammation. In the first chapter of this thesis, a general literature view on this hypothesis is presented.

Observations in a series of bowel-related joint diseases gives support to this hypothesis: in Crohn's disease (CD) and ulcerate colitis, the bowel wall inflammation is complicated by joint inflammation symptoms in 5-23% of the cases. In bypass disease, which may follow the construction of a blind loop in the small bowel to promote weight loss, an arthritis occurs in about 20% of the cases, probably due to bacterial overgrowth in the blind loop. The finding that bowel infection by *Salmonella*, *Shigella* and *Yersinia* species may be complicated by joint inflammation symptoms also supports an etiological link between bowel bacteria and arthritis.

The contents of the large bowel is characterized by the presence of a high number of microorganisms: up to 2 x 10^{11} bacteria per g faeces occur, and more than 99% of them are strictly anaerobic. The major cell wall component of Gram-positive bacteria is peptidoglycan; this amino sugar polymer has potent inflammatory properties such as the activation of complement and stimulation of lymphocytes and macrophages. Fragments of this peptidoglycan macromolecule are liberated during bacterial growth and division. It is supposed that these fragments pass the bowel wall during the lifelong exposure and are taken up by the immune system and transported to the joints and other tissues. As these bacterial cell walls are poorly degradable, a disturbance between influx and breakdown may cause peptidoglycan complexes to cumulate in joint tissues.

In 1977, Cromartie *et al.* described an animal model for the study of arthritis. They isolated cell wall fragments (CWF) from *Streptococcus pyogenes*. After a single intraperitoneal injection of these cell walls, rats developed an acute polyarthritis of paw joints, followed by a chronic phase characterized by exacerbations and remissions. We adopted this model to study the arthropathic properties of CWF isolated from Gram-positive anaerobic bacteria of the human intestinal flora.

In the stool of patients with CD, Gram-positive anaerobic coccoid rods are found more frequently and in higher numbers than in the stool of healthy individuals. To study the role of the bowel flora in the joint complications in CD, we isolated CWF from four anaerobic Gram-positive coccoid rods. CWF from two of these, both being *Eubacterium contortum* strains, induced chronic arthritis in Lewis rats. These data are described in

Chapter 2.

In Chapter 3 we describe the arthropathic properties of CWF from nine representative Gram-positive strains from the human anaerobic intestinal flora, strains which are present in high numbers (10^8-10^9 per g faeces, or even higher) in the stool of healthy individuals. We found a great variety in arthropathic properties, ranging from a persistent chronic arthritis (*Eubacterium aerofaciens* and *Bifidobacterium* species) to an absence of joint inflammation symptoms (*Eubacterium rectale, Lactobacillus leichmanii* and *Clostridium* species) after cell wall administration. This diversity was also seen when several species of the bacterial genus *Eubacterium* were studied (Chapter 4). Only minor differences in arthropathic properties were found between four isolated *E. aerofaciens* strains (Chapter 6).

From studies using CWF of *Lactobacillus* species and *S. pyogenes* it has been hypothesized that the presence of cell wall rhamnose is necessary to induce a chronic joint inflammation. Another cell wall property, the *in vitro* cell wall resistance to lysozyme, is supposed to contribute to the persistence of CWF in tissues, leading to chronic joint inflammation. The large number of bacterial species investigated in our studies permitted us to test both hypotheses; our experimental results, however, did not support either hypothesis. CWF of the lysozyme resistant *C. ramosum*, for example, failed to induce chronic arthritis, even in the presence of cell wall rhamnose (Chapter 3), whereas CWF from *E. aerofaciens* RA1, sensitive to *in vitro* digestion by lysozyme and without cell wall rhamnose, induced chronic joint inflammation after a single intraperitoneal injection (Chapter 6). It is not clear which factors account for the arthropathic properties of bacterial cell walls.

The potency of *E. aerofaciens* CWF to induce chronic joint inflammation was histologically confirmed (Chapter 5). The acute phase was characterized by tissue oedema, synovial lining proliferation and infiltration of synovial tissue by polymorphonuclear granulocytes, macrophages and some lymphocytes. In the chronic phase the synovial infiltrate contained more lymphocytes and less granulocytes; marginal cartilage and bone erosions as well as apposition of new bone tissue were characteristic features. In some rats, an active synovitis was observed up to 90 days after cell wall injection. This *E. aerofaciens* CWF-induced arthritis has many aspects in common with that induced by *S. pyogenes* and *S. faecium* CWF. Furthermore, several features are shared with RA in man, as the synovial infiltration, proliferation of synovial lining cells and marginal erosions of cartilage and bone. However, some dissimilarities were also found: in bacterial cell wall-induced arthritis, synovial plasma cells and follicle-like structures are almost absent, in contrast to RA.

Another way to study the involvement of the anaerobic intestinal flora in RA is to investigate whether or not the resident faecal flora of RA patients differs from that of healthy individuals. We found *Eubacterium* and *Bifidobacterium* present in the flora of both RA patients and healthy subjects; the number of *Eubacterium* and *Bifidobacterium* species did not differ, but the flora of 10 RA patients contained significantly more

anaerobic coccoid rods than the flora of 10 healthy individuals (Chapter 6). The impact of this increase in anaerobic coccoid rods in RA is not clear; an altered intestinal flora might be an expression of a genetic predisposition to RA. We also isolated *E. aerofaciens* strains from the intestinal flora of RA patients and healthy individuals; CWF from all 4 strains isolated induced chronic arthritis in the rat after intraperitoneal injection.

We conclude that the intestinal flora of healthy individuals and patients with RA harbour anaerobic bacteria with a wide range of arthropathic properties in the rat. These observations give experimental evidence to the hypothesis that the anaerobic intestinal flora is involved in the induction or maintenance of chronic joint inflammation. As our experiments have been performed in a rat model, and the bacterial cell walls used in our studies probably are not identical to bacterial cell wall compounds liberated in the intestine, our results have to be interpreted carefully regarding to RA in man. Chemical and arthropathic properties of bacterial cell wall compounds isolated from intestinal contents have to be compared to those of CWF from cultured bacteria. In addition, studies on the occurrence of bacterial cell wall compounds in joint tissues of RA patients may provide valuable experimental evidence that the bacterial load from the bowel is involved in chronic joint inflammation in man.

SAMENVATTING

Reumatoïde artritis (RA) komt voor bij 1-2% van de bevolking en heeft als belangrijkste kenmerk een chronische steriele ontsteking van een of meer gewrichten; de oorzaak is niet bekend. Al een aantal decennia wordt gedacht aan een microbiële oorzaak, maar een specifieke verwekker kon tot dusver niet overtuigend worden aangetoond. Een hypothese is, dat bestanddelen van darmbacteriën een rol spelen bij het in gang zetten of onderhouden van chronische gewrichtsontstekingen bij de mens. In het eerste hoofdstuk van dit proefschrift wordt vanuit de literatuur deze hypothese toegelicht.

Klinische waarnemingen waarbij een verband wordt gezien tussen gewrichtsontstekingen en darminfectie of ontsteking van de darm geven steun aan deze hypothese: bij een deel van de patiënten met de ziekte van Crohn of colitis ulcerosa wordt de chronische ontsteking van de darmwand gecompliceerd door gewrichtsontstekingen. Ook patiënten met vetzucht, bij wie de dunne darm kortgesloten is om gewichtsverlies te bevorderen, kunnen lijden aan gewrichtsontstekingen. Men vermoedt, dat bacteriële overgroei in de darmlis die buiten gebruik is gesteld, de oorzaak is van de gewrichtsontstekingen. Bij een klein deel van de patiënten met een acute darminfectie door *Salmonella-*, *Shighella-* of *Yersinia*-bacteriën treedt tevens een gewrichtsontsteking op.

De inhoud van de dikke darm bestaat voor ruim 1/3 uit bacteriën; van de meer dan honderdduizend miljard bacteriën die voorkomen in één gram ontlasting, leeft meer dan 99% in een zuurstofloos milieu (= anaeroob). Een belangrijk bestanddeel van de celwand van bacteriën is peptidoglycaan: om de celmembraan heen ligt dit stevige netwerk van ketens van aminosuikers, die onderling verbonden zijn door aminozuren. Peptidoglycaan heeft ontstekingsbevorderende eigenschappen, zoals de stimulatie van witte bloedcellen en de activatie van complement. Groeiende en delende bacteriën scheiden peptidoglycaanfragmenten uit; verondersteld wordt, dat deze bacteriële celwandbestanddelen de darmwand passeren, door het afweersysteem opgenomen worden en ook in de gewrichten terecht komen. Omdat de blootstelling aan dit slecht afbreekbare peptidoglycaan zeer langdurig is, kan het zich in gewrichten ophopen, met name in het geval van een verstoorde aanvoer of afbraak van dit bacteriële materiaal.

In 1977 werd een proefdiermodel voor de studie van gewrichtsontstekingen beschreven. Celwandfragmenten van de bacterie *Streptococcus pyogenes* werden in een eenmalige dosis in de buikholte van Lewis-ratten geïnjecteerd; de ratten ontwikkelden daarop acute gewrichtsontstekingen aan de poten, gevolgd door chronische ontstekingen die herhaaldelijk opvlamden en uitdoofden. We hebben dit model gebruikt om te onderzoeken of celwandfragmenten van anaerobe darmbacteriën in staat zijn om gewrichtsontstekingen op te wekken.

In de darmflora van patiënten met de ziekte van Crohn komt een bepaalde groep bacteriën, de groep van de anaerobe coccoïde staafjes vaker en in hogere aantallen voor dan bij gezonden. Om te bestuderen of deze bacteriën betrokken zijn bij de gewrichtsontstekingen die bij de ziekte van Crohn voorkomen, hebben we celwanden van vier coccoïde staafjes uitgetest in het proefdiermodel. Het bleek dat celwanden van twee *Eubacterium contortum*-stammen een chronische gewrichtsontsteking in de rat konden opwekken (hoofdstuk 2).

Hoofdstuk 3 beschrijft het uittesten in het proefdiermodel van celwanden van negen bacteriesoorten die representatief zijn voor een belangrijk deel van de menselijke darmflora. We vonden grote verschillen: *Eubacterium aerofaciens* en *Bifidobacterium*-soorten konden een chronische gewrichtsontsteking opwekken, terwijl celwanden van *Eubacterium rectale*, *Lactobacillus leichmanii* en *Clostridium*-soorten geen ontstekingsverschijnselen opriepen. Ook tussen een aantal soorten binnen één bacteriegeslacht (*Eubacterium*) vonden we grote verschillen in het vermogen om gewrichtonstekingen op te wekken, variërend van geen, alleen een acute of chronische, tot een acute en chronische gewrichtsontsteking (hoofdstuk 4). Vier stammen van dezelfde bacteriesoort (*E. aerofaciens*) bleken weinig te verschillen in hun vermogen om gewrichtsontstekingen op te wekken (hoofdstuk 6).

Ook werd de relatie bestudeerd tussen eigenschappen van bacteriële celwanden en hun vermogen om een langdurige gewrichtsontsteking op te wekken. In de literatuur over het gewrichtsontsteking-inducerende vermogen van celwanden van *Lactobacillus*-soorten en *S. pyogenes* wordt de hypothese gesteld, dat de aanwezigheid van rhamnose in de bacteriële celwand nodig is om een langdurige gewrichtsontsteking op te wekken. Een tweede hypothese stelt dat bacteriële celwanden resistent moeten zijn tegen lysozyme om een langdurige gewrichtsontsteking te kunnen opwekken. Lysozyme is een van de weinige enzymen die in staat is de aminosuikerketens van het peptidoglycaan te knippen en zo een essentiële bijdrage kan leveren aan de afbraak van peptidoglycaan in weefsels. Wordt het peptidoglycaan in weefsels slecht afgebroken, dan is een langdurige ontsteking het gevolg. Onze experimentele bevindingen met een groot aantal bacteriestammen geven geen steun aan beide hypothesen. Zo geeft bijvoorbeeld *E. aerofaciens* stam RA-1 een chronische gewrichtsontsteking, ondanks een goede gevoeligheid voor lysozyme en de afwezigheid van rhamnose (hoofdstuk 6). *Clostridium ramosum* daarentegen leidt ondanks lysozyme-resistentie en een hoog gehalte aan rhamnose niet tot ontstekingsverschijnselen (hoofdstuk 3). Het is niet duidelijk welke celwandeigenschappen bepalen of de celwanden van een bepaalde bacterie wel of niet een langdurige gewrichtsontsteking kunnen opwekken.

Het vermogen van *E. aerofaciens* celwanden om een langdurige gewrichtsontsteking op te wekken werd ook histologisch bevestigd (hoofdstuk 5). De acute fase van de gewrichtsontsteking werd gekenmerkt door weefseloedeem, proliferatie van de synoviale bekleding en infiltratie van synoviaal weefsel door granulocyten, macrofagen en een aantal lymfocyten. In de chronische fase bevatte het synoviale weefsel meer lymfocyten

en minder granulocyten. Op de overgang van synovia naar kraakbeen kwamen erosies van kraakbeen en onderliggend bot voor en op botvlies werd afzetting van nieuw bot gezien. Op dag 90 na celwandinjectie was er bij een deel van de ratten nog een aktieve ontsteking van de synovia waarneembaar. De gewrichtsontstekingen die werden opgewekt door celwandfragmenten van *E. aerofaciens*, leken sterk op de gewrichtsontstekingen die ontstonden na injectie van *S. pyogenes*- en *Streptococcus faecium*-celwanden. Er is een aantal overeenkomsten met RA bij de mens, zoals infiltratie van synovium door lymfocyten en marginale erosies van bot en kraakbeen. Er zijn echter ook verschillen: in de gewrichtsontstekingen die worden opgewekt door bacteriële celwandfragmenten, ontbreken de synoviale plasmacellen en folliculaire lymfocyten aggregaten vrijwel geheel; dit in tegenstelling tot wat bij RA het geval is.

Vervolgens werd nagegaan of de anaerobe darmflora van patiënten met RA verschilde van die van gezonden (hoofdstuk 6). Uit de darmflora van beide groepen werden *Eubacterium*- en *Bifidobacterium*-soorten geïsoleerd. De totale aantallen *Eubacterium*- en *Bifidobacterium*-soorten verschilden niet, maar wel werd in de darmflora van een groep van tien RA-patiënten een hoger aantal anaerobe coccoïde staafjes gevonden dan in de darmflora van tien gezonden. De betekenis van deze toename in aantallen coccoïde staafjes bij RA is niet bekend; mogelijk is de afwijkende darmflora een uiting van een erfelijke aanleg voor RA. Ook werden uit de darmflora van twee RA patiënten en twee gezonden *E. aerofaciens*-stammen geïsoleerd die in het proefdiermodel een langdurige gewrichtsontsteking konden opwekken.

Ter afsluiting kan gezegd worden dat in de darmflora van zowel gezonden als patiënten met RA anaerobe bacteriestammen voorkomen die een grote verscheidenheid aan gewrichtsontstekingen in de rat kunnen opwekken. Deze waarnemingen ondersteunen de hypothese dat de anaerobe darmflora betrokken is bij het ontstaan of onderhouden van chronische gewrichtsontstekingen bij de mens. Omdat de experimenten in een proefdier gedaan zijn en omdat de bacteriële celwanden die in deze studies gebruikt zijn waarschijnlijk niet identiek zijn aan celwandfragmenten die door groeiende en delende bacteriën in de darminhoud uitgescheiden worden, moeten onze bevindingen met voorzichtigheid geïnterpreteerd worden. Celwandfragmenten van gekweekte bacteriën en celwandfragmenten geïsoleerd uit faeces zouden moeten worden vergeleken wat betreft de chemische samenstelling en het vermogen om gewrichtsontstekingen op te wekken. Daarnaast zou onderzoek naar het vòòrkomen van bacteriële celwandbestanddelen in gewrichtsweefsel van patiënten met RA steun kunnen opleveren voor de hypothese dat de darmflora betrokken is bij chronische gewrichtsontstekingen bij de mens.

LIST OF ABBREVIATIONS

AS	ankylosing spondylitis
ATCC	American Type Culture Collection
CD	Crohn's disease
CFA	complete Freund adjuvant
CWF	cell wall fragments
EDTA	ethylenediaminetetraacetic acid
GF	germfree
HLA	human leukocyte antigen
HS	healthy subjects
i.a.	intra-articular
IL	interleukin
IBD	inflammatory bowel disease
i.p.	intraperitoneal
IFA	incomplete Freund adjuvant
i.v.	intravenous
kD	kiloDalton
LPS	lipopolysaccharide
MDP	muramyldipeptide
MHC	major histocompatibility complex
NSAID	non-steroid anti-inflammatory drug
PBS	phosphate buffered saline
PEG	polyethylene glycol
PG	peptidoglycan
PG-PS	peptidoglycan-polysaccharide
PMN	polymorphonuclear cells
PS	polysaccharide
PY	peptone yeast extract medium
RA	rheumatoid arthritis
s.c.	subcutaneously
SCW	streptococcal cell walls
SF	synovial fluid
SPF	specific pathogen-free
UC	ulcerative colitis

DANKWOORD

Dit proefschrift is, hoewel er slechts één auteursnaam op de kaft staat, niet het werk van een enkeling. Velen hebben bijgedragen aan het werk dat zijn neerslag vindt in dit boekje, en een aantal wil ik met name noemen.

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