INDUCTION OF CHRONIC ARTHRITIS IN RATS

THE ROLE OF INTESTINAL BACTERIA AND BACTERIAL CELL WALL FRAGMENTS

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Kool, Jeanette

Induction of chronic arthritis in rats : the role of
intestinal bacteria and bacterial cell wall fragments /
Jeanette Kool. - [S.l. : s.n.] (Alblasserdam : Haveka).-
Ill.
Proefschrift Rotterdam. - Met lit. opg. - Met samenvatting
in het Nederlands.
ISBN 90-73436-08-7
NUGI 743
Trefw.: gewrichtsontstekingen / darmflora.

INDUCTION OF CHRONIC ARTHRITIS IN RATS
THE ROLE OF INTESTINAL BACTERIA AND BACTERIAL CELL WALL FRAGMENTS

INDUCTIE VAN CHRONISCHE GEWRICHTSONTSTEKINGEN IN DE RAT
DE ROL VAN DARMBACTERIËN
EN BACTERIËLE CELWAND FRAGMENTEN

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof. dr. C.J. Rijnvos
en volgens besluit van het college van dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 3 juni 1992 om 13.45 uur

door

Jeanette Kool
geboren te Hilversum.

Promotiecommissie:

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Dit proefschrift werd bewerkt binnen de afdeling Immunologie van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam.

De publikatie van dit proefschrift werd mede mogelijk gemaakt door een subsidie van het Nationaal Reumafonds.
Publication of this thesis was supported by the Dutch Rheumatism Foundation.

Omslag: macrofagen in de milt, aangekleurd met een monoclonale antistof tegen bacteriële celwand fragmenten.

Het proefschrift werd gedrukt door Haveka B.V. te Alblasserdam.

Voor mijn ouders
Aan Martin

CONTENTS

CHAPTER 1

RHEUMATIC DISEASES AND RHEUMATOID ARTHRITIS

1.1 INTRODUCTION

1.1.1 Rheumatic diseases

The rheumatic diseases are a heterogeneous group of diseases, involving the locomotory system, that in the past were seen as one disease entity. The word "rheumatism" is inherited from the humoral pathology, as described extensively by Galen (129-195 AD). In the humoral pathology disease is explained as a disbalance of the four body fluids (1): black bile (melan chole), yellow bile (chole), blood (sanguis) and slime (phlegma). Each fluid was thought to be produced by an organ; slime was thought to come from the brain. If there was an overproduction of slime it would flow downward (kata-rheo in Greek), leading to catarrhal diseases or rheumatism. Two characteristics that were connected to slime were "cold" and "wet" (figure 1), thus rheumatism was correlated with cold and wet weather.

Nowadays disease entities involving the joints are separately recognized, for example gout, osteoarthrosis, rheumatic fever, ankylosing spondylitis, reactive arthritis and rheumatoid arthritis. These disease entities have a different cause, a different therapy is needed and also the prognostic outcome differs largely.

Several of the arthritic diseases are related to bacterial infections or bacterial products from the intestine. Rheumatic fever is an acute disease involving the joints and the heart. The arthritis is caused by a cross-reactivity between the M-protein of group A streptococci and the articular cartilage and synovium of the joint (2). The existence of cross-reactivity was also shown between the M-protein and myocardial tissue (3,4). The arthritic disease is mostly self-limiting, but the attack on the myocardial tissue induces irreversible damage. Therefore infections with these bacteria (e.g., angina) and entering of the bloodstream by these bacteria (e.g., after tooth extraction) must be prevented by antibiotic treatment in patients once having suffered from rheumatic fever.

Reactive arthritis is an arthritis especially affecting the joints of the lower limbs and is seen after a gastrointestinal infection with *Salmonella*, *Shigella*, *Yersinia* or *Campylobacter*. Bacteria could not be cultured from the joint (5), nor could bacterial DNA be detected in the joint fluid by polymerase chain reaction (6), indicating that viable bacteria are not present in synovial fluid of reactive arthritis patients. Granfors *et al.*, however, demonstrated the presence of *Yersinia* (7) or *Salmonella* (8) antigens in synovial fluid cells. Therefore it is thought that reactive arthritis is caused by the deposition of bacterial debris (forming immune complexes with antibacterial antibodies) in the joint.

Reiter's syndrome consists of reactive arthritis and conjunctivitis related to urethritis caused by *Chlamydia trachomatis*. *Chlamydia* antigens could be demonstrated in the synovial fluid cells. No viable *Chlamydia* were present in synovial fluid (9), nor could *Chlamydia* DNA be detected in synovial fluid or tissue by polymerase chain reaction (10).

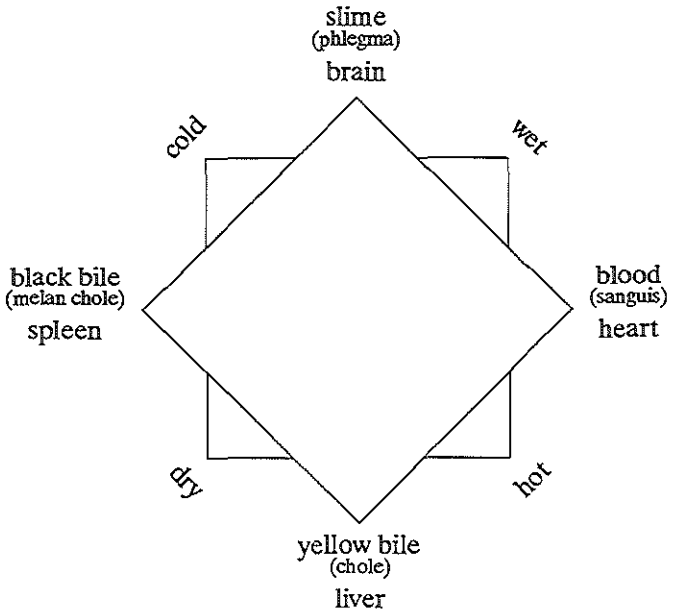

Figure 1. Humoral pathology according to Galen/Hippocrates.

A specific T cell response to *Chlamydia trachomatis* probably plays a role in the pathogenesis of Reiter's syndrome (11).

Reactive arthritis after gastrointestinal as well as genitourinary infection is linked to the major histocompatibility complex class I molecule HLA-B27. Of the patients suffering from Reiter's syndrome 63-75% are HLA-B27 positive (12). The arthritis is usually self-limiting, but sometimes a chronic relapsing course may develop (13).

In ankylosing spondylitis, or Morbus Bechterew, predominantly the lower spinal column and sacroiliacal joints are involved. This disease, like reactive arthritis, is linked to HLA-B27, but a stronger relationship is found. More than 90% of the ankylosing spondylitis patients are HLA-B27 positive (12). As in reactive arthritis a relation with gastrointestinal bacteria, in this case *Klebsiella* and *Yersinia*, has been established. It is, however, not thought to be the result of local deposition of debris as in reactive arthritis. Three hypothesis have been proposed. In the first hypothesis the disease is thought to be based on a cross-reactivity between the nitrogenase enzyme of *Klebsiella* or the surface membrane protein of *Yersinia* and HLA-B27 (14). The second is the altered-self hypothesis: a modifying factor produced by *Klebsiella* induces the expression of epitopes on HLA-B27 positive cells only (15). This epitope cross-reacts with an epitope on a limited number of enteric bacteria (certain strains of *Escherichia coli*, *Salmonella*, *Shigella*

and *Klebsiella*). Why especially the lower spinal column is involved is not explained by these two hypotheses. The third hypothesis suggests that a self-peptide, occurring only in the joints involved in ankylosing spondylitis, binds to HLA-B27 with a higher affinity than to other HLA molecules. An infection with bacteria, sharing epitopes with this self-peptide, would initiate the inflammation of the spinal column (16). Ankylosing spondylitis as well as reactive arthritis is seen more in men than in women (ratios 3:1 and 9:1, respectively, (12)).

1.1.2 Rheumatoid arthritis

Rheumatoid arthritis is a chronic disease with polyarthritis as the major symptom, but extraarticular symptoms also occur (anemia, lung diseases). The joint inflammations often occur symmetrically, i.e. in the same joints of the left and right half of the body. The joints that are most frequently involved are the interphalangeal, metacarpophalangeal and wrist joints, followed by the knee, elbow and feet joints. Swelling, pain and morning stiffness are major complaints. These serve as diagnostic criteria together with the presence of rheumatoid nodules, rheumatoid factor and radiographic erosions and/or osteopenia (17). About 1% of the population suffers from rheumatoid arthritis (18,19). Women are more prone to develop rheumatoid arthritis than men, the ratio being 2-3:1. The highest incidence is found at the age of 30-50 years (19). In Caucasians and Japanese the disease is genetically linked to the major histocompatibility complex class II molecule HLA-DR4 (20), or more specifically Dw4 and Dw14 in Caucasian and Dw15 in Japanese (21,22). In Swiss and Jews rheumatoid arthritis is linked with HLA-DR1 (22), which shares the involved epitope with Dw14 (23). Calin *et al.* (24) suggested that the presence of HLA-DR4 leads to a more severe form of rheumatoid arthritis instead of conferring a higher relative risk to develop rheumatoid arthritis. Also HLA-DQ (DQw7) has been correlated with an increased risk of rheumatoid arthritis (25), but it is possible that the linkage of HLA-DQw7 with HLA-DR4 is responsible for this correlation (22).

Rheumatoid arthritis is an important health problem. It has been suggested that about 20% of patients may experience a complete remission (18). Another study showed, however, that remissions do not last long (26). Rheumatoid arthritis causes pain and disability in the individual patient, leading to a loss of effectively spent time of 32% (27). Also the mortality rate is increased. In 20% of the patients rheumatoid arthritis is the direct cause of death and in another 20% rheumatoid arthritis-related conditions are involved in death, leading to a shortened life-expectancy of 15 and 10 years, respectively (28). In rheumatoid arthritis patients whose death is not directly or indirectly related to rheumatoid arthritis, life-expectancy is shortened by 5 years (28). Infections, renal diseases and cardiovascular diseases are the most frequent causes of death in rheumatoid arthritis patients (29).

Therapy in rheumatoid arthritis patients (30,31) is largely symptomatic. Drug treatment usually begins with nonsteroid antiinflammatory drugs. These often have only a limited effect and a second line (disease modifying antirheumatic) drug is added, for example antimalarials, gold compounds, penicillamine or sulfasalazine. In refractory rheumatoid arthritis third line drugs are given; cytotoxic (azathioprine, methotrexate, cyclophosphamide) or immunosuppressive (prednisone, cyclosporine A) agents can be used. Finally, experimental therapies can be used, for example ductus thoracicus drainage and total lymphoid irradiation.

The influence of the diet on rheumatoid arthritis has long been, and still is, a subject of discussion. Recently some indications for an effect of a fish oil diet has been published (32,33). Also in an animal model of arthritis evidence was found for a positive effect of fish oil on the severity of arthritis (34). Kjeldsen-Kragh *et al.* reported on the positive effect of elimination of animal products from the diet of rheumatoid arthritis patients (35).

In 1991 a report on the consumption of health care by rheumatoid arthritis patients in the Netherlands was published (18). General practitioners are consulted at least 159.000 times a year and specialists (including rheumatologists) at least 140.000 times a year. About 4900 rheumatoid arthritis patients stay in hospital for a mean of 25 days and some 1000 patients stay in health care institutes for a mean of 360 days. This indicates that apart from an individual health problem rheumatoid arthritis is also an important care-consuming problem that costs the community at least Dfl 158,000,000.-/year.

The third problem related to rheumatoid arthritis is the work disability. Ten years after the onset of arthritis 50% of the patients are unable to work, after 15 years this is increased to 67% (26). Also household work disability in women with moderate-to-severe rheumatoid arthritis is substantial (36).

The exact cause of rheumatoid arthritis is not known. It has been suggested that viable microorganisms (bacteria, viruses, mycoplasmata) may be involved. However, microorganisms have only occasionally been isolated from inflamed joints in rheumatoid arthritis, although regularly raised titers against for example Epstein-Bar virus (37) or *Proteus* (38) have been found. Nowadays many investigators do not think that an infection is the cause of rheumatoid arthritis. There are, however, several observations which point to a role for microorganisms or their products, especially those derived from the intestinal tract:

- Up to 40% of patients with Crohn's disease and up to 26% of patients with ulcerative colitis experience arthritis of the peripheral joints (39). The arthritic complaints can be alleviated by colectomy in ulcerative colitis patients, in patients with Crohn's disease remission after colectomy is less certain (40).
- Jejunal bypass surgery for obesitas is often (up to 20% of the patients) complicated by arthritis, which is thought to be based on bacterial overgrowth of the blind loop, resulting in increased production and uptake of bacterial fragments (41-43). The

arthritis is usually self-limiting, but sometimes restoration of the normal intestinal anatomy is needed.
- Ankylosing spondylitis is linked to increased numbers of *Klebsiella* in the intestine.
- In reactive arthritis and Reiter's disease antigens derived from the microorganism causing the intestinal cq urethral infection have been demonstrated in synovial fluid cells.
- Cell wall fragments of aerobic (44) and anaerobic bacteria (45-48) from the human intestine are arthritogenic in an animal model.

1.2 HISTOLOGY OF RHEUMATIC JOINTS

There are two main reasons to perform histological examination of joint specimens. First, histology is used as a diagnostic means to confirm that someone has rheumatoid arthritis. Discrimination from other kinds of arthritis is, however, difficult. Second, histology is used to study the pathogenesis of rheumatoid arthritis. The latter especially applies to animal studies. In both cases it is important to know the normal anatomy and histology of the joint.

The involved joints in rheumatoid arthritis are mostly diarthrodial, i.e., formed between two long bones. The joint is formed by the ends of both bones that are covered with hyalin cartilage consisting for 70% of water with collagen fibers (20%) intermingled with proteoglycans (5-7%) and harboring isolated chondrocytes (49). At the edge of the joint the cartilage becomes thinner and meets the synovial membrane which covers the inside of the joint capsule. This capsule is made of tough fibrous tissue and is surrounded by ligaments and tendons. It gives the joint stability as well as liberty of action. Surrounded by synovium and articular cartilage is the joint space filled with fluid that serves as a lubricant for the joint, but also supplies the non-vascularized cartilage with nutrients. In this synovial fluid normally no cells are present.

The most important structure in rheumatoid arthritis is the synovial membrane. It normally consists of a 1-3 cells thick superficial layer of endothelial-like cells (synovial lining) and of synovial tissue. About 30% of the synovial lining cells are class II (HLA-DR) positive cells showing acid phosphatase activity and expressing macrophage antigens and Fc-receptors (50,51). This so-called type I cell (52) is phagocytic and stems from the monocyte-macrophage lineage. The other 70% of the synovial lining cells are class II negative, acid phosphatase negative, macrophage-antigen negative, fibroblast-antigen positive cells (50,51): the fibroblast-like type III cell as described by Burmester *et al.* (52). This cell type is non-phagocytic and produces hyaluron, the lubricant of the synovial fluid.

In the synovial tissue the fibroblast predominates. This cell type produces the hyaluron and collagen of the matrix of the synovial tissue. Sometimes HLA-DR positive, acid phosphatase positive cells are found perivascularly (51). Other leukocytes that sporadically

may be present are T lymphocytes, also mostly perivascularly (53). B cells are normally not found in the synovial tissue (54).

At the synovio-cartilage junction the synovium overlies the cartilage for about 1 cm forming a kind of pannus, with a lining of predominantly fibroblast-like type III cells and a highly vascularized fibrous tissue beneath the lining. In this tissue macrophages are found adjacent to the bone, but no lymphocytes are present (55).

1.2.1 Histology of rheumatoid arthritis

Rheumatoid arthritis starts as an early synovitis with edema and a polymorphonuclear infiltrate. As no basal membrane is present beneath the synovial lining these polymorphonuclear cells rapidly pass into the synovial fluid. Apart from the predominating polymorphonuclear cells also T lymphocytes, B lymphocytes and macrophages are found in the synovial tissue, with numbers of T cells exceeding plasma cells. In a later phase the synovitis becomes more proliferative in character (56,57). Synovial lining cells and fibroblasts in the synovial tissue proliferate, resulting in a hypertrophic synovium with enlarged villi. The lining layer consists of 30-50% type I cells (HLA-DR positive, macrophage-antigen positive cells) and about 30% type II cells (HLA-DR positive, macrophage-antigen negative non-phagocytic cells (49,52,58)). The latter are not present in normal synovial lining. This results in a higher overall class II expression of the synovial lining compared with normal joints. The type III (class II negative, macrophage-antigen negative, fibroblast-antigen positive) cell is found in a layer directly under the lining layer (58). These cells are also highly proliferative. In the synovial tissue HLA-DR positive cells can be found, mostly in a perivascular distribution (59,60). These cells are often found in connection with T helper cells (60-63). T suppressor/cytotoxic cells are less numerous than T helper cells and are found more equally distributed over the synovial tissue (60,62). The ratio of T helper to T suppressor/cytotoxic cells can vary from 4:1 to 14:1 (64,65), with only one study reporting a predominance of T suppressor/cytotoxic cells over T helper cells (66). B cells or plasma cells are present in smaller numbers than T cells (62,67). They are mostly IgM (62) positive but also IgG positive cells are found (68).

In chronic rheumatoid arthritis erosion of the bone and cartilage is a major characteristic. The proces starts at the synovio-cartilage junction, where granulomatous tissue that is continuous with the synovium, enters the bone via enlarged vascular foramina (57). The cortical bone is eroded and the cartilage, which is very resistant to erosion, is undermined. Pannus, normally present for maximally 1 cm at the synovio-cartilage junction, spreads further over the cartilage. Thus the cartilage is attacked from two sides and eventually is also eroded. In the last stage of rheumatoid arthritis the cartilage is replaced by fibrous tissue, which leads to ankylosis of the joints.

In describing the histopathology of rheumatoid arthritis many problems are encountered. First, it is not always known for how long the arthritis has existed before histology was done. In many studies arthritis existed more than one year before histologic investigation was performed (53,54,61,69). Second, it is suggested that large differences exist in histological findings within a joint (70). Thus it might be important to take biopsies under arthroscopic view at the most inflamed sites of the joint. Other studies (71,72), however, contradict this: no large differences exist within the same joint in patients that have not received medication. Third: what is the correlation between histology and clinical activity of the disease? There seems to be a positive correlation between synovial hyperplasia and disease activity only in patients not receiving medication. In another report, however, histologic abnormalities, like hyperplasia and perivascular mononuclear infiltrate, were even found in clinically uninvolved knees of rheumatoid arthritis patients (73). The fourth problem (related to previously mentioned problems) is the influence of medication on the histological findings. This has long been neglected. In a recent study Haraoui *et al.* (60) investigated the effects of prednisone, methotrexate and nonsteroid antiinflammatory drugs on the histology of rheumatoid arthritis. Prednisone and methotrexate increased synovial lining hyperplasia and mononuclear infiltrate (especially T helper cells) compared with nonsteroid antiinflammatory drugs, but decreased the extent of fibrosis. As reported by Moore *et al.* (74) aurothiomalate and penicillamine decreased the number of T helper cells. The last problem is the specificity of the histological changes for rheumatoid arthritis: many of the described histopathological characteristics can also be found in non-rheumatoid synovitis. In osteoarthrosis, where the synovitis is secondary to the cartilage destruction, also synovial hyperplasia with increased HLA-DR expression is found as well as high numbers of T lymphocytes, predominantly T helper cells, perivascularly situated together with HLA-DR positive cells (75). The differences are gradual: the cellular component is less pronounced, the fibrosis more pronounced compared with rheumatoid arthritis. Also in ankylosing spondylitis a strong T cell infiltrate has been described, although in ankylosing spondylitis the T helper to T suppressor ratio was 1:1 (69). Barkley *et al.* (76) described an identical occurrence of B cells, T cells and HLA-DR/DP expression for rheumatoid arthritis and reactive arthritis. In rheumatoid arthritis, however, a higher HLA-DQ expression, more macrophage-antigen positive cells and more synovial lining hyperplasia were found. Lindblad (53) demonstrated that synovitis and non-rheumatoid arthritis differed from rheumatoid arthritis only in severity of the histological findings: synovial lining proliferation, perivascular T cell infiltration (with T helper cells exceeding T suppressor/cytotoxic cells) and perivascular HLA-DR positive cells were found in all types of synovitis, but were most prominent in rheumatoid arthritis. The only pathognomonic finding in rheumatoid arthritis might be the granulomata: follicle-like structures of HLA-DR positive cells, T helper cells and plasma cells (56). They occur, however, in only 2% of the patients.

1.2.2 Histology of experimental arthritides

Two rat models in which arthritis is induced by injection of bacterial material have been described by other investigators. The histology of both models as well as the histology of arthritis in our own model will be described in this paragraph.

The first model is the adjuvant-induced arthritis model as described by Pearson (77). He also wrote the most extensive report on the histological findings (78), which were confirmed by Stanescu *et al.* (79). About 2 weeks after subcutaneous injection of 0.5 mg *Mycobacterium tuberculosis* in oil an arthritis of front and hind paws develops. Histologically the arthritis starts with edema, followed in 2-4 days by exudation into the joint space of neutrophils and deposition of fibrin. At the same time proliferation of the synovial lining (normally 1-2 cell layers) and mononuclear/fibroblastic infiltrate of the synovial tissue occurs. A mononuclear/fibroblastic infiltrate is also found along muscles and tendons. Between 5 and 10 days after the first signs of arthritis development the neutrophilic exudate in the joint space is replaced by a mononuclear/macrophage exudate. The cellular infiltration of synovial tissue has increased. Granulomatous tissue, continuous with inflamed synovium, erodes subchondral bone at the synovio-cartilage junction and overlies the cartilage as pannus. Sometimes periosteal new bone formation is seen. After 11 to 20 days the pannus and new bone formation has increased. The periarticular infiltrate now consists predominantly of lymphocytes; sometimes plasma cells are present. Between 21 and 30 days, granulation tissue invades the marrow spaces and spreads subchondrally. Pannus extends over the cartilage eroding or replacing it. A predominantly lymphocytic infiltrate is found in synovial tissue and foci of active fibroblasts together with foci of metaplastic cartilage are seen. Bone apposition is less active than before, with a rim of immature cartilage. From day 31 on invasion of the bone by granulation tissue and extension of pannus over the cartilage goes on in a less aggressive way, but still leading to destruction of the cartilage and ultimately to fibrous ankylosis. The synovial reaction subsides, although hyperplasia of the lining and a lymphocytic infiltrate are still present. A few plasma cells are also found. Periarticular new bone formation sometimes leads to bony ankylosis. More than 60 days after the start of arthritis signs of active inflammation are seldom found and the chronic reaction is less aggressive.

In the second model, first described (also histologically) by Cromartie *et al.* (80), arthritis is induced by intraperitoneal injection of about 20 mg of streptococcal cell wall fragments suspended in buffer. The histological findings in this model were extensiated by Dalldorf *et al.* (81) and Allen *et al.* (82). Within 1 day after injection edema and a neutrophilic infiltrate are found perivascularly in the synovial tissue. The joint space is filled with neutrophils and fibrin. At day 3 the inflammatory reaction is most intense with fibrin deposition in synovial tissue and joint space, synovial lining hyperplasia, villus formation and infiltration of synovial tissue with neutrophils and mononuclear cells.

Tendons, joint capsule, periarticular tissue and subcutaneous tissue are also infiltrated with neutrophils and mononuclear cells. At day 12 neutrophils have disappeared completely, but lining hyperplasia, villus formation and mononuclear infiltration are still present. Proliferation of fibroblast-like cells in synovial tissue is found. From 2 weeks on lining hyperplasia, villus formation, neutrophilic exudation in the joint space and mononuclear infiltration of synovial tissue increases further to reach a maximum at 84 days. Tendons, joint capsule and subcutaneous tissue are not involved in the inflammation in this chronic phase. Vascular proliferation is found from day 15 on and also pannus is first seen at day 15. Considerable erosion of subchondral bone and destruction of cartilage is seen from day 21 and day 42 on, respectively. Subperiosteal new bone formation appears around day 30. Eventually fibrous ankylosis evolves (day 60 and later).

The second model has been used by our group to study the arthritogenicity of anaerobic intestinal bacteria (45-48). The histological characteristics, using *Eubacterium aerofaciens* cell wall fragments to induce arthritis, have been described by Severijnen *et al.* (83). Two days after injection edema, a polymorphonuclear infiltrate of synovial tissue, and a polymorphonuclear exudate in the joint space is found. Substantial synovial lining hyperplasia occurs from day 4 on. Periarticular infiltration and tendosynovitis is found from day 5 till day 21. Between 2 and 3 weeks after injection the predominance of polymorphonuclear cells in synovial tissue is taken over by lymphocytes. Macrophages and fibroblast-like cells are seen, especially near bone and cartilage erosions. No plasma cells or mast cells are present. Subchondral bone erosions are first found 12 days after injection. At the same time new bone formation appears. Bone apposition and erosions become more frequent at later timepoints. In the chronic phase pannus tissue extended into the joint space. Contrary to the adjuvant-induced arthritis model, active inflammation occurs even 60 days after induction of arthritis in this model.

The histological features of the arthritis show only minor differences between the rat models. The differences are probably not related to the model used (as will be shown in chapter 4), but are more likely to be the result of a stochastic proces. The differences between the rat models and human rheumatoid arthritis are somewhat larger: in the rat models practically no plasma cells are present. In human rheumatoid arthritis plasma cells are present, although always in lower numbers than T lymphocytes. Another feature not encountered in rat models, but present in human rheumatoid arthritis, is osteoporosis. Still the similarities dominate: a neutrophilic infiltrate followed by a lymphocytic infiltrate, with a predominance of T helper cells over T suppressor/cytotoxic cells; hyperplasia of the synovial lining and hypertrophic synovial villi; granulomatous tissue invading bone marrow, and pannus overlying the cartilage, eventually leading to erosion of bone and cartilage; ultimately fibrous tissue replaces the cartilage and ankylosis evolves.

1.3 T LYMPHOCYTES AND ARTHRITIS

1.3.1 T lymphocytes in human rheumatoid arthritis

There are several clinical indications that T lymphocytes play an important role in rheumatoid arthritis. One of the first reports on this subject appeared in 1977. Paulus *et al.* (84) removed the lymphocytes by thoracic duct drainage and in this way was able to improve grip strength, ring size, morning stiffness and number of tender joints in 9 rheumatoid arthritis patients. Reinjection of the removed lymphocytes resulted in a relapse in some patients, as did cessation of the thoracic duct drainage in almost all patients.

In a later study, using lymphapheresis, Yeadon and Karsh (85) showed that removal of $1\text{-}2\text{x}10^{10}$ lymphocytes improved rheumatoid arthritis clinically, without changing lymphocyte count or % B or T cells in the blood. Wahl *et al.* (86) also demonstrated improvement after lymphapheresis, especially in those patients showing anergy to soluble antigens (eg. tetanus toxoid, streptokinase, streptodornase) before lymphapheresis. Improvement was not related to lymphopenia or immunosuppression as tested by delayed type hypersensitivity reaction, but anergy was abolished after lymphapheresis.

Total lymphoid irradiation improved rheumatoid arthritis clinically in 24 of 32 patients (87). The improvement was maximal 6 months after total lymphoid irradiation and remained stable until 48 months after total lymphoid irradiation in most patients. In this study total lymphocytes, but especially CD4 positive cells, as well as responsiveness of peripheral blood mononuclear cells to allogeneic and mitogenic stimulation were reduced.

Cyclosporin A treatment resulted in a decrease in disease activity in 6 of 9 rheumatoid arthritis patients. No change in number of leukocytes or cell subtypes was present, nor in stimulation assays with mitogen (phytohaemagglutinin, concanavalin A) or antigen (streptokinase, *Candida*, purified protein derivative of tuberculin, (88)).

Another indication for the role of T lymphocytes is the beneficial effect of antithymocyte globulin reported in 2 of 3 rheumatoid arthritis patients (89). The total lymphocyte count was decreased, but number of T cells, especially CD8 positive cells was increased.

The most recent clinical study concerning involvement of T cells was done by Reiter *et al.* (90). Treatment of rheumatoid arthritis patients with anti-CD4 resulted in improvement of rheumatoid arthritis, as assessed by Ritchie's articular index, pain, grip strength and morning stiffness, with concomitant decrease of peripheral blood CD4 positive cells.

These studies point to a role for T cells in rheumatoid arthritis, although it is not clear whether the clinical effect is based on a change in numbers of T lymphocytes (or T cell subsets), or on a change in T lymphocyte function, or on both.

Not only clinical studies, but also many in vitro experiments on T lymphocytes in

rheumatoid arthritis have been done. Both the phenotype and function of T cells isolated from rheumatoid arthritis patients have been investigated. Holoshitz *et al.* (91) found 11% γδ T cells in rheumatoid arthritis synovial fluid compared with 4% in rheumatoid arthritis peripheral blood. (In peripheral blood of healthy subjects 5-10% γδ T cells are present, (92)). Especially the Vδ1 gene is used more frequently among synovial fluid γδ T cells compared with peripheral blood T cells (93), based on a polyclonal expansion of synovial fluid T cells expressing Vδ1 (94). The majority of T cells, however, express the αß T cell receptor.

From synovial fluid, T cells can be isolated with a CD4:CD8 ratio up to 2:1 (95,96). From synovial tissue a higher proportion of CD4 positive cells can be isolated (97-100), which is consistent with the phenotype of T cells as assessed histologically in rheumatoid synovia (see chapter 1.2.1). The association of rheumatoid arthritis with HLA-DR4 and HLA-DR1, restriction elements for CD4 positive T cells, also indicates a role for T helper lymphocytes. In synovial fluid as well as synovial tissue T lymphocytes with the $CD4^+CDw29^+$ phenotype (T helper cells stimulating B cells: the T helper-inducer cell) are more numerous than $CD4^+2H4^+$ cells (T helper cells that induce T suppressor/cytotoxic cells: the T suppressor-inducer cell, (95,96,100)). CDw29+ T cells were shown to adhere to the fibroblast-like synoviocytes (101).

Rheumatoid arthritis mononuclear cells are also functionally different:

- Mononuclear cells from the synovial fluid proliferate spontaneously in medium without interleukin 2, mitogen or antigen (102,103).
- Mononuclear cells from the synovial fluid and from peripheral blood show a higher proliferative response in the presence of interleukin 2 than peripheral blood mononuclear cells from healthy subjects (102,104).
- Mononuclear cells from the synovial fluid show a lower proliferative response in the presence of mitogen (phytohaemagglutinin, pokeweed mitogen, anti-CD3) compared with control or rheumatoid arthritis peripheral blood mononuclear cells (102,103,105).
- A higher frequency of autoreactive T cells is found in synovial fluid compared with control peripheral blood lymphocytes (autologous mixed lymphocyte reaction, (104)). This autologous mixed lymphocyte reaction is HLA-DR driven and interleukin 2 dependent (106). A proliferative response on syngeneic chondrocytes has also been described (107).
- A higher proliferative response on soluble antigens (such as mycobacterial 65 kD (heat shock protein), *Salmonella*, *Yersinia*, *Candida* and acetone-precipitated *Mycobacterium tuberculosis*) in synovial fluid compared with rheumatoid arthritis peripheral blood is found (103,105,108,109).

Many authors have tried to demonstrate clonal dominance among synovial T cells. But until now no unequivocal oligoclonality has been established. From synovial tissue, T cells could be isolated that after propagation in the presence of interleukin 2 showed limited oligoclonality on Southern blot analysis of T cell receptor DNA (98,109). Duby *et al.*

(111) and van Laar *et al.* (112), also using Southern blot analysis, found the same pattern of T cell receptor rearrangements in rheumatoid arthritis synovial fluid or synovial tissue as in rheumatoid arthritis or normal peripheral blood. Using monoclonal antibodies specific for Vß families Brennan *et al.* (113) demonstrated an increased usage of Vß5 and Vß8 by rheumatoid arthritis synovial T cells compared with rheumatoid arthritis peripheral blood lymphocytes. Paliard *et al.* (114) showed an increase in Vß14 usage in rheumatoid synovium compared with peripheral blood, based on a decrease in Vß14 usage in rheumatoid arthritis peripheral blood compared with normal peripheral blood. Uematsu *et al.* (115), applying an inverse polymerase chain reaction, was able to investigate the T cell receptor repertoire of rheumatoid arthritis synovial mononuclear cells without prior expansion of cells in the presence of interleukin 2. Although both Vα and Vß usage were heterogeneous, a 2-3 fold increase of Vß2.1 and Vß3.1 usage compared with rheumatoid arthritis peripheral blood could be demonstrated.

Although some clonal dominance among synovial T cells compared with peripheral blood T cells is found by several authors, the results do not point to a single specific clone involved in rheumatoid arthritis, since each author finds another type of clonal dominance. The majority of the T lymphocyte population in rheumatoid arthritis synovium is heterogeneous in T cell receptor V gene usage.

1.3.2 T lymphocytes in experimental arthritides

In the experimental arthritides evidence for a role for T cells has accumulated. In the adjuvant-induced arthritis model arthritis could be transferred by lymph node, spleen, or thoracic duct cells (116-118); however, Quagliata and Quagliata (119) suggested that arthritis induction in these cases was based on carry-over of antigen. More direct evidence implicating the T cells in adjuvant-induced arthritis was presented by Holoshitz *et al.* (120) who could transfer the disease with a CD4 positive T cell clone.

In the streptococcal cell wall-induced arthritis model, apart from histological evidence (see chapter 1.2.2), a report comparing athymic and euthymic rats demonstrated the role of T lymphocytes in the development of the chronic phase of arthritis (82). Recently transfer of arthritis by a CD4 positive T cell line in this model was reported by DeJoy *et al.* (121). Also in our model of arthritis induced by injection of *Eubacterium aerofaciens* cell wall fragments the disease could be transferred by a CD4 positive αß T cell line (122).

In the adjuvant-induced arthritis model as well as the streptococcal cell wall-induced arthritis model modulation of arthritis can be achieved by treatment with drugs or monoclonal antibodies. Cyclosporin A inhibited streptococcal cell wall-induced arthritis (123). Cyclophosphamide reduced the severity of adjuvant-induced arthritis in a susceptible rat strain, but it potentiated arthritis in a resistent rat strain, which coincided

with a decrease in CD8 positive T cells in the peripheral blood of these rats (124,125).

Elimination of T helper cells, by treatment with anti-CD4 monoclonal antibody, or of T cells with the αß T cell receptor prevents the development of arthritis and causes suppression of established arthritis in both the adjuvant-induced arthritis model and the streptococcal cell wall-induced arthritis model (126-128). Treatment with anti-CD8 monoclonal antibody resulted in responsiveness to adjuvant-induced arthritis in tolerated rats (129).

Summarizing it may be said that the results of investigations on T cells in human rheumatoid arthritis as well as experimental arthritides suggest the involvement of CD4 positive T cells (probably the $CDw29^+$ helper-inducer subset) in the development and maintenance of chronic arthritis. CD8 positive T cells that normally perform a regulatory role, are probably deficient in number or function in arthritis.

1.4 BACTERIAL ANTIGENS IN MAMMALIAN TISSUES

Several arthritides (reactive arthritis, ankylosing spondylitis) are related to intestinal bacteria; and intestinal diseases (Crohn's disease, ulcerative colitis, jejunal bypass syndrome) can be complicated by arthritis. This led to the supposition that rheumatoid arthritis may also be related to the intestinal flora. Several authors hypothesized that bacterial fragments, derived from the intestinal flora, may pass the bowel wall, distribute over the body and cause a local or systemic reaction leading to arthritis (130-133).

Proof for this hypothesis has come mainly from animal studies. For instance, cell wall fragments from several human anaerobic intestinal Gram-positive bacteria induced severe, chronic arthritis when injected intraperitoneally in rats (45-48).

Systemic absorption of bacterial cell wall polymers was demonstrated after gavage feeding and after intracecal injection of streptococcal peptidoglycan-polysaccharides (134,135). In serum of rats with a jejunal bypass, an increased titer of anti-peptidoglycan antibodies was found, indicating that a higher intraluminal bacterial load (bacterial overgrowth of the blind loop), resulted in an increased uptake of peptidoglycan by an intact intestine (136). After intraluminal injection of peptidoglycan-polysaccharides these could be found in the plasma of injected rats (136). On the other hand, no muramyl peptides could be detected in normal serum of rabbits (137), although muramic acid was readily detected up to a concentration of 125ng/ml after intravenous injection of muramyl peptides.

In adjuvant-induced arthritis as well as in streptococcal cell wall-induced arthritis the presence of bacterial antigen in the joints has been demonstrated after subcutaneous injection of *Mycobacteria* (138-140) and after intraperitoneal injection of *Streptococci* (81,141-143), respectively, indicating that a *local* immune reaction may be the cause of the joint inflammation.

Patient studies until now render contradicting results. The anaerobic intestinal flora of rheumatoid arthritis patients does not differ from the anaerobic intestinal flora of healthy subjects (48), although other authors described an increased number of *Clostridium perfringens* (144,145). Sapico *et al.* (146) and Struthers (147) could not confirm the increase in *C.perfringens* in the intestinal flora of rheumatoid arthritis patients. In aerobic species no differences were shown.

The intestinal permeability as measured by ethylenediaminetetraacetic acid or ß-lactoglobulin uptake, is higher in rheumatoid arthritis patients than in healthy controls, but this is probably due to the use of nonsteroidal antiinflammatory drugs by rheumatoid arthritis patients (148-152). Ethylenediaminetetraacetic acid and ß-lactoglobulin uptake is also increased in controls taking nonsteroid antiinflammatory drugs (152,153). Studies on polyethyleneglycol 400 permeability gave contradicting results: increased as well as decreased uptake has been reported (154-156). A study on sugar uptake showed no change in rheumatoid arthritis patients versus healthy subjects (157). The molecules used in these patient studies (polyethyleneglycol 400, ethylenediaminetetraacetic acid, sugars) are relatively small (MW < 400) compared with bacterial cell wall fragments (MW about 10^5).

Indirect evidence for an increased permeability for bacterial cell wall polymers in rheumatoid arthritis patients comes from serum-antibody studies. Raised titers of anti-peptidoglycan-polysaccharide of *Streptococci* have been found in rheumatoid arthritis patients compared with controls (158). But in other studies no raised anti-peptidoglycan, anti-mucopeptide or anti-D-ala-D-ala titers could be demonstrated (159-161). In control sera, antibody titers to mucopeptide could be found in 22% of the subjects (160).

Studies on the presence of bacterial cell wall components in rheumatoid arthritis joints have been inconclusive: muramic acid (a component specific for bacterial cell walls) could not be detected with a mass spectrometry procedure (162). With a similar procedure muramic acid was only detectable in synovial fluid of septic arthritis patients with high bacterial counts, indicating that mass spectrometry may be too insensitive to detect muramic acid in rheumatoid arthritis (163).

No clearcut proof of a changed intestinal flora or an increased intestinal permeability in rheumatoid arthritis patients has been provided so far. However, it may be possible that bacterial cell wall polymers are able to pass the bowel wall in healthy subjects as well as rheumatoid arthritis patients, but that passage results in an (adverse) immunological reaction in rheumatoid arthritis patients. As described above, there are indications that peptidoglycan-polysaccharide polymers from the intestinal flora pass the bowel wall (titers in normal sera, peptidoglycan-polysaccharide polymers in plasma after intraluminal injection). Further proof comes from an animal study in which muramic acid, a compound specific for bacterial cell walls, could be demonstrated in the liver, spleen and brain of conventional rats (164).

1.5 INTRODUCTION TO THE EXPERIMENTAL WORK

We have investigated the hypothesis that bacterial cell wall fragments pass the bowel wall and induce an immune response leading to arthritis. We have adopted two animal models: adjuvant-induced arthritis (77) and streptococcal cell wall-induced arthritis (80). Both models have been used before by several investigators to study bacteria-induced arthritis; *Mycobacterium tuberculosis* and *Streptococcus pyogenes* are the arthritogenic agents in the respective models. In many respects the adjuvant-induced arthritis model and the streptococcal cell wall-induced arthritis model are similar (histologically, chapter 1.2.2; role of T cells, chapter 1.3.2). The streptococcal cell wall-induced model, however, differs from the adjuvant-induced arthritis model in that an acute, complement-dependent, arthritis develops within 2 days after intraperitoneal injection, before the onset of chronic arthritis (165).

In previous experiments Severijnen *et al.* (45-48) have used the streptococcal cell wall-induced model to investigate the arthritogenicity of anaerobic Gram-positive bacteria from the human intestinal flora. Also the histology (hematoxylin and eosin staining) in this model has been described (83). In chapter 2 of this thesis an immunohistological investigation of the leucocyte subsets in arthritic paws of rats injected with *Eubacterium aerofaciens* cell wall fragments is described. Neither in the adjuvant-induced arthritis model nor in the streptococcal cell wall-induced arthritis model a systematic inventarisation of cell types involved in the chronic phase of arthritis has been performed.

The influence of the germfree status on arthritis-susceptibility has been described for susceptible Lobund rats (166) as well as for resistent Fischer rats (167,168) in the adjuvant-induced arthritis model and the streptococcal cell wall-induced arthritis model: germfree rats appeared to be susceptible to arthritis induction. In chapter 3 the influence of decontamination on arthritis induction by intraperitoneal injection of *Eubacterium aerofaciens* cell wall fragments (intraperitoneal model) in susceptible Lewis rats was studied. The occurrence of arthritogenic species among the autochthonous flora of the Lewis rat was also investigated.

In the streptococcal cell wall-induced arthritis model only relatively large size fragments (i.e. $>5x10^7$ dalton) are able to induce chronic arthritis (169). In the adjuvant-induced arthritis model, however, also smaller fragments can induce arthritis. A second advantage is the lower dose of antigen (1 mg) that is sufficient to induce arthritis in this model compared with the streptococcal cell wall-induced arthritis model (20 mg). Therefore the arthritogenicity of cell wall fragments of *Eubacterium* species after subcutaneous injection in oil (subcutaneous model) was investigated and compared with the intraperitoneal model (chapter 4).

In chapter 5 the arthritogenicity of smaller sized (i.e. $<10^5$ dalton) peptidoglycan-polysaccharide complexes, that are derived from the intestinal flora was studied. Soluble peptidoglycan-polysaccharide complexes were isolated from faeces or ileostomy fluid and

injected in the subcutaneous model. The enzymic activity in ileostomy fluid, which may be an important factor influencing the arthritogenicity of peptidoglycan-polysaccharide complexes, was also studied. The results of this study are described in chapter 6.

One of the main suppositions in our hypothesis is the passage of bacterial cell wall fragments over the bowel wall. As mentioned in chapter 1.4 there is only limited evidence for the presence of bacterial peptidoglycan in the tissues of normal rats; it is not known which cells contain the bacterial products. In chapter 7 the production of a monoclonal antibody against peptidoglycan-polysaccharide complexes is described and this monoclonal antibody was used to investigate the presence of bacterial fragments in the tissues of normal rats. The distribution of target structures of this antibody within these tissues is also described.

In the last chapter I will discuss the results described in the chapters 2-7.

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CHAPTER 2

IMMUNOHISTOLOGY OF JOINT INFLAMMATION INDUCED IN RATS BY CELL WALL FRAGMENTS OF *EUBACTERIUM AEROFACIENS**

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* *Submitted for publication*

SUMMARY

After a single intraperitoneal injection of cell wall fragments of *Eubacterium aerofaciens*, a main resident from the human intestinal flora, an acute arthritis develops within two days which is followed by a chronic arthritis that lasts at least 90 days. In an earlier report the histological appearance of the joint inflammation during this period has been described. In this study we investigated in more detail the cell types that are involved in the development of arthritis by using cell type specific monoclonal antibodies in an immunohistological assay. In the acute phase of arthritis T helper cells appeared in the synovial tissue together with ED1 positive and ED3 positive macrophages. After a temporary decline at day 12 all macrophage subsets as well as T helper cells reappeared/increased again at day 33. Later in the chronic phase (day 47-90) an increased number of ED1 positive cells in the synovial tissue and a decreased number of ED2 positive cells in the synovial lining compared with control rats was the most prominent finding. These results indicate that apart from T lymphocytes also macrophages play an important role in the development and continuation of chronic arthritis in this model.

INTRODUCTION

Several authors have proposed an etiological role for microbial agents in rheumatoid arthritis. To investigate this hypothesis two rat models using bacteria or bacterial products are currently used. The first model is the adjuvant-induced arthritis model, in which *Mycobacterium tuberculosis* whole cells suspended in Freund's incomplete adjuvant are injected subcutaneously and give rise to a chronic polyarthritis after 2-3 weeks (1). Also cell wall fragments from *M. tuberculosis* and several other species appeared to be arthritogenic in this model (2-5). In the second model streptococcal cell walls suspended in phosphate buffered saline are injected intraperitoneally (6). This induces an acute (day 1-14) followed by a chronic (from day 15 on) joint inflammation. *Lactobacillus* cell wall fragments have been shown to be arthritogenic as well in this model (7).

In the past we have directed our investigations towards the human intestinal flora, especially the numerous Gram-positive anaerobic bacteria. Cell wall fragments of several species of *Eubacterium* and *Bifidobacterium* were arthritogenic after intraperitoneal injection in buffer (IP model, ref. 8-10) or after subcutaneous injection in oil (SC model, ref. 2). Also bacteria-derived peptidoglycan-polysaccharide complexes directly isolated from intestinal contents proved to be able to induce chronic joint inflammation (11).

Both in the adjuvant-induced arthritis model and the streptococcal cell wall-induced arthritis model several reports have been published that describe the histological pattern

of these arthritides. The most extensive study in the adjuvant-induced arthritis model has been performed by Pearson and Wood (12) who described the histology of arthritic paws, spinal column and tail from the first day of occurrence of arthritis until one year later. Stanescu *et al.* (13) studied ankle joints in the adjuvant-induced arthritis model over the same period of time. Both studies, however, are limited to conventional histology: no immunohistologic determination of cell (sub-)types was performed.

In the streptococcal cell wall-induced arthritis model similar conventional histology studies have been done during the acute and chronic phase of the arthritis by Cromartie *et al.* (6). Until now the only immunohistochemical study in the streptococcal cell wall-induced arthritis model has been done by Allen *et al.* (14). This study, however, was limited to one timepoint (week 6) of the arthritis and only the occurrence of T cell (subsets) was investigated. They found clusters of T cells around vessels within the synovium with a predominance of T helper lymphocytes over T suppressor/cytotoxic cells.

In an earlier report we described the conventional histology of *Eubacterium aerofaciens* cell wall fragments induced arthritis (IP model) during the acute and chronic phase (15). In the acute phase edema and a polymorphonuclear infiltrate of the synovium predominated. In the chronic phase the major cell types were lymphocytes and macrophages. No mast cells or plasma cells were found in synovial tissue. In the present report we further differentiate between the cell types present in arthritic ankle joints of *E. aerofaciens* cell wall fragments injected rats at several time points during the acute and chronic phase of arthritis with an immunohistochemical method using a set of monoclonal antibodies directed against rat leukocytes. The results are related to observations in human rheumatoid arthritis as well as results from arthritis models in the rat.

MATERIALS AND METHODS

Animals

Female Lewis rats (Harlan Sprague Dawley, Bicester, UK) weighing 140-170 gram were used. A group of 16 rats were given a single intraperitoneal injection of *Eubacterium aerofaciens* cell wall fragments (CWF) suspended in phosphate buffered saline. Each rat received a dose of CWF equivalent to 25 μg muramic acid per gram body weight. Four non-injected rats served as controls.

During the observation period (0-90 days) the diameters of wrists and ankles were measured with a vernier caliper at regular intervals. The severity of the arthritis was expressed as the sum diameter of wrists and ankles.

Bacteria

E. aerofaciens ATCC 25986 was obtained from the American Type Culture Collection (Rockville, MD) and was cultured overnight at 37°C on Schaedler broth (Oxoid Ltd, London, UK) under strictly anaerobic conditions after inoculation with a log phase culture. Bacterial CWF were prepared as has been described previously by Severijnen *et al.* (16). Muramic acid and rhamnose contents of the CWF were determined as has been described by Hadzija (17) and Dische and Shettles (18) respectively. The isolated *E. aerofaciens* CWF contained 15% (dry weight) muramic acid (corresponding with 60% peptidoglycan, the arthritogenic component of the CWF) and 29% rhamnose.

Preparation of tissue blocks

At days 2, 5, 12, 21, 33, 47, 65 and 90 after cell wall injection 2 rats were sacrificed for immunohistologic examination. After cardiac puncture bleeding under ether anaesthesia the right hind paws were skinned, embedded in 8% (w/v) gelatin (Merck, Darmstadt, Germany) and slowly frozen in liquid nitrogen. The tissue blocks were stored at -70°C until sectioning. Four control rats were processed in the same way.

Preparation of cryostat sections

The procedure described by van Noorden and Vogels (19) was used. In short: the frozen paws were cut on a motor-driven Bright type 5030 cryostat with a tungsten carbide tipped knife at -28°C. Sagittal sections of 10 μm thickness were cut, attached to adhesive tape (Scotch Brand 800, 3M, St Paul, MN) and fixed with waterproof adhesive tape onto slides. The sections were kept at -20°C until staining.

Staining procedure

The thawed sections were fixed with hexazotized pararosaniline as described by de Jong *et al.* (20). In short 1 ml 4% (w/v) pararosaniline-hydrochloride (Sigma, St Louis, MO) in 2M HCl and 1 ml 4% (w/v) $NaNO_2$ was mixed and 194 ml distilled water was added after 1 minute. Sections were fixed for 2 minutes and directly afterwards washed for 10 minutes in phosphate buffered saline (0.01M, pH=7.4) with 0.2% bovine serum albumin (PBS/BSA). It was not necessary to inhibit endogenous peroxidase, as no endogenous activity was found.

The mouse anti-rat monoclonal antibodies (MoAb, table 1) diluted in PBS/BSA were

Table 1. Monoclonal antibodies used in the staining of ankle joints.

MoAb	Specificity	Reference
B115-5	CD5 antigen, all T lymphocytes, autoimmune B cells	23
B115-4	CD4 antigen, T helper lymphocytes, macrophages, monocytes	23
OX8	CD8 antigen, T suppressor lymphocytes	24
His14	B lymphocytes	25
OX6	Ia antigen (non-polymorphic)	26
ED1	monocytes, macrophages, dendritic cells	27
ED2	resident macrophages	27
ED3	macrophage subpopulation in lymphoid organs	27
NS7	sheep red blood cells	28
AcPh	acid phosphatase activity	22

incubated overnight at 4°C in a humidified chamber. Control slides were incubated with PBS/BSA or the control monoclonal antibody NS7. As second step antibody peroxidase conjugated rabbit immunoglobulin to mouse immunoglobulins (Dakopatts, P260, Denmark) was used in a 1:250 dilution in PBS/BSA with 1% normal rat serum to minimize background staining.

After 1 hour incubation at 4°C the substrate was added for 10 minutes at room temperature. As substrate 3,3'-diaminobenzidine-tetrahydrochloride (Sigma) 0.05% (w/v) dissolved in 0.1M acetate buffer (pH 6.0) with Imidazole (0.07% w/v, Merck), $NiSO_4.6H_2O$ and 0.01% H_2O_2, as described by Green *et al.* (21) was used. Positive cells are stained black.

Acid phosphatase (AcPh) activity was demonstrated by incubating the slides with a solution of 0.05% (w/v) naphthol AS-BI phosphate (Sigma), 0.04% pararosaniline and 0.04% $NaNO_2$ in 0.1M acetate buffer (pH=5.0) during 30 minutes at room temperature (22).

Sections were evaluated independently by two of the investigators (JK and MG) and results were expressed as - (no positive cells present) to +++ (maximal number of positive cells present). Maximal number of positive cells are defined as number of positive cells as stained by ED2 at day 33 in the periarticular tissue. Staining of all cells (100% positive cells) was never found. The results obtained by the two investigators never differed more than one degree with each other.

Monoclonal antibodies

Table 1 lists the monoclonal antibodies used in this study. OX6 (MCA 46) and OX8

(MCA 48) were purchased from Serotec (Oxford, UK). B115-4 and B115-5 were purchased from Holland Biotechnology (Leiden, The Netherlands). ED1, ED2 and ED3 were kindly provided by Dr C.D. Dijkstra (Free University, Amsterdam, The Netherlands). HIS14 was kindly provided by Dr F.G.M. Kroese (State University, Groningen, The Netherlands). NS7 (ATCC TIB114) was from the American Type Culture Collection.

RESULTS

Arthritis induction

Figure 1 shows the arthritis pattern of 6 rats (from the total of 16 rats) that were observed for 47-90 days. One of these rats failed to develop any inflammatory symptoms, all other 15 rats injected developed an arthritis. This rat was excluded from the immunohistological study at day 65. Control rats showed no clinical signs of joint inflammation.

Figure 1. Arthritis induction by cell wall fragments from *Eubacterium aerofaciens*. Each line represents a single rat. Only rats observed for at least 47 days are shown. Sum paw diameter of control rats varied between 21.5 and 22.5 mm.

Table 2. Occurrence of various cell types in ankle joints of control and arthritic rats*

	ED1	ED2	ED3	Ia	CD5	CD4	CD8	His14	AcPh
Control joints									
synovial lining	+	+++	−	+	−	−	−	−	++
synovial tissue	−	++	−	++	±	±	−	−	−
periarticular	−	++	−	+	−	−	−	−	−
day 2									
synovial lining	±	−	−	−	−	−	−	−	−
synovial tissue	+	++	−	±	−	−	−	−	±
periarticular	++	++	−	+	±	−	−	−	+
day 5									
synovial lining	+	+	+	−	±	±	−	−	+
synovial tissue	++	++	+	++	++	+	−	−	+
periarticular	++	+	−	+	±	+	−	−	++
day 12									
synovial lining	−	−	−	−	−	−	−	−	+
synovial tissue	±	+	±	±	−	−	−	−	+
periarticular	+	++	+	+	±	−	±	−	++
day 21									
synovial lining	±	±	±	−	−	−	−	−	+
synovial tissue	+	+	±	+	±	±	±	−	±
periarticular	+	+	±	+	±	±	−	−	+
day 33									
synovial lining	+	++	++	+	+	−	−	−	++
synovial tissue	+	++	+	++	++	+	−	±	+
periarticular	+	+++	+	++	+	+	±	±	+
day 47									
synovial lining	+	+	−	+	−	−	−	−	++
synovial tissue	+	+	−	++	−	±	−	−	+
periarticular	+	++	−	++	−	−	−	−	±
day 65									
synovial lining	+	+	−	±	−	+	−	−	++
synovial tissue	+	++	−	++	−	±	−	−	+
periarticular	±	+	−	+	−	−	−	−	±
day 90									
synovial lining	+	±	−	±	−	−	−	−	++
synovial tissue	++	++	−	++	−	+	−	−	+
periarticular	−	++	−	+	−	−	−	−	−

* Number of cells expressed as − = no cell stained to +++ = maximal number of cells stained as described in materials and methods.
Results are presented as the mean of two rats (except for day 65: results of one rat). The control group consisted of four rats.

Immunohistology

The results of the immunohistochemical staining are summarized in table 2. The differences, if present, between the two rats of each group were 1 degree at its most.
Control joints. Both in synovium and periarticular tissue many cells were stained by ED2. Also major histocompatibility complex class II (Ia) expression was found in synovium and periarticular tissue but in a lower number of cells. ED1 staining and AcPh activity was only found in the synovial lining. Sporadically CD4 positive T lymphocytes were present in synovial tissue. No ED3 positive macrophages or HIS14 positive B cells were seen.
Arthritic joints. At *day 2* a decrease in ED1 positive and ED2 positive cells in synovial lining was found. In contrast, an increase in ED1 positive and ED2 positive cells in synovial tissue was seen. Also in the periarticular tissue many ED1 and ED2 positive cells were found. Ia expression had diminished in the synovial tissue as well as synovial lining. Some cells in the synovial tissue showed AcPh activity, but no AcPh activity was found in the synovial lining. Neither ED3 positive cells nor B or T lymphocytes were found.

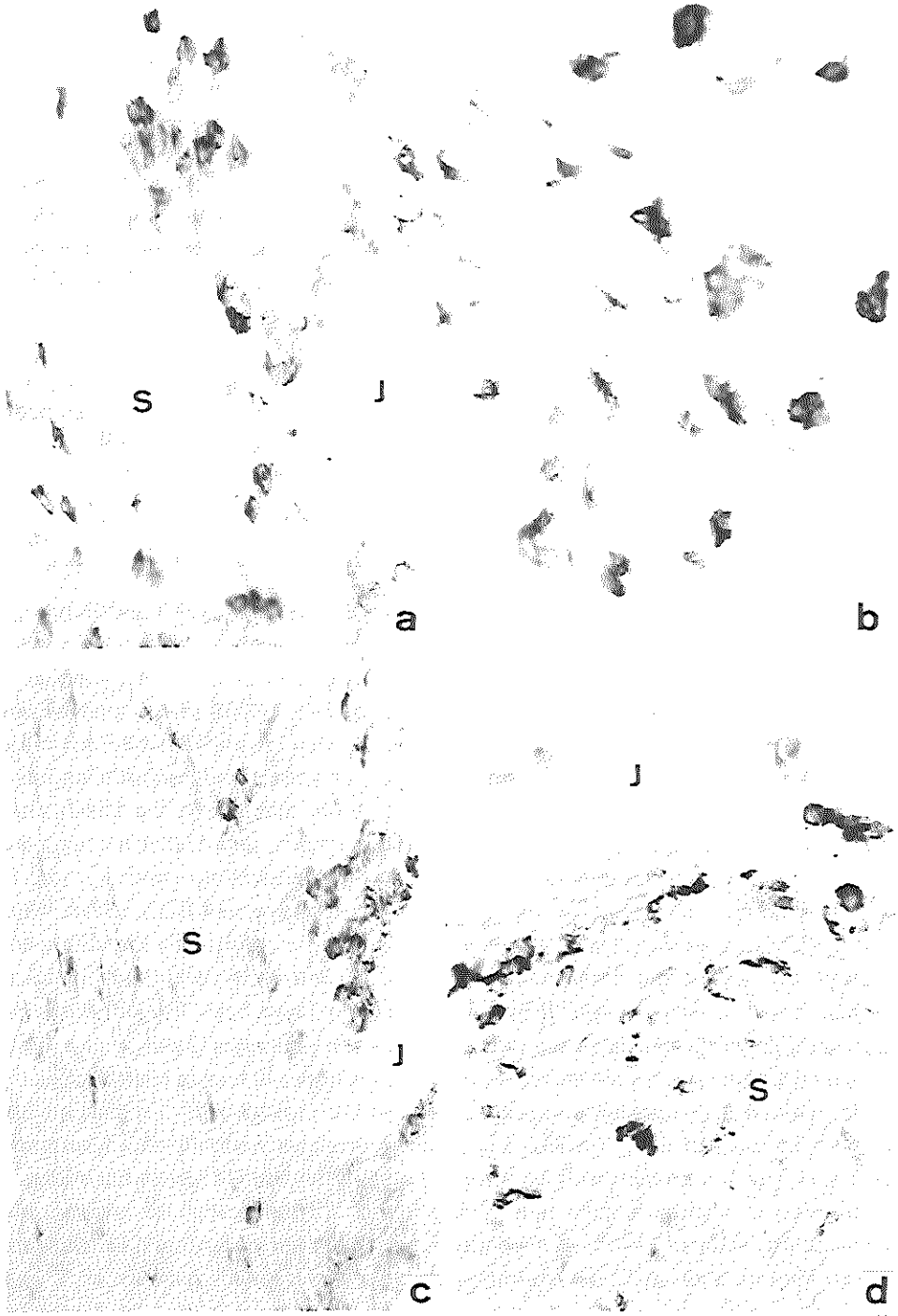
At *day 5* the number of ED1 positive cells in the synovial tissue increased further, while the number of ED2 positive cells remained stable. At this timepoint the first ED3 positive cells were found in synovial tissue as well as in the lining. In the lining also ED1 positive and ED2 positive cells were present. Ia expression and AcPh activity was shown in synovial tissue and periarticular tissue. AcPh activity was additionally found in synovial lining. Many CD5 positive T lymphocytes were found in the synovial tissue, and sporadically in the synovial lining. All of these T cells were CD4 positive. B lymphocytes were not present.

At *day 12* almost no CD5 positive cells were present. In the synovium the number of macrophages compared with day 5 had also decreased, but periarticularly the ED2 positive and ED3 positive cells had increased. Especially in synovial tissue Ia expression had diminished compared with day 5. Again B cells were absent.

Sections of *day 21* rats showed a limited number of ED1 positive, ED2 positive and ED3 positive cells in synovial tissue and lining. Ia expression was found only in the synovial and periarticular tissue, while AcPh activity was present in tissue and lining. A small number of CD5 positive T lymphocytes, predominantly of the T helper subset, were present in the synovial tissue.

After *33 days* the number of CD4 positive T cells (figure 2a) in the synovial tissue had

Figure 2. Immunohistology of arthritic rats 33 days (a,b,c) and 90 days (d) after injection with cell wall fragments from *Eubacterium aerofaciens*. (a) CD5 positive T lymphocytes (monoclonal antibody B115-5) are present in the synovial tissue and, in smaller numbers, in the synovial lining. (b) ED2 positive cells are found in the synovial tissue. (c) In the synovial lining ED3 positive cells are present. (d) ED1 positive cells are still present 90 days after injection of cell wall fragments. S = synovium, J = joint cavity. (Magnification: 225 x).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increased again compared with day 21 as well as ED2 positive cells (figure 2ab and Ia expression. In the synovial lining and the connective tissue all macrophage subsets (figure 2c), Ia expression and AcPh activity had risen. This was the only timepoint when a limited number of His14 positive B lymphocytes was seen in the synovial tissue.

From *day 47* on no ED3 positive cells were observed. The number of ED2 positive cells remained stable in synovial tissue, but decreased in synovial lining. The number of ED1 positive cells also remained stable (figure 2d). Ia expression was fairly high in synovial tissue, but low in synovial lining, while AcPh activity showed the reverse distribution. T lymphocytes had considerably diminished compared with day 33 and B lymphocytes were not found any more.

At this chronic phase of arthritis granuloma-like structures were found in the synovium, with many AcPh positive cells. Either ED1 positive or ED3 positive cells were present, together with T lymphocytes, but without ED2 positive macrophages or B lymphocytes. Ia expression varied from - to + + + in the various granuloma-like structures.

DISCUSSION

The control rats in our study did not stain positively with ED3, OX8 and His14. Some CD5 positive cells were found dispersed in the synovial tissue. As we found no staining with His14, these CD5 positive cells were not B lymphocytes but T lymphocytes. We found weak staining with ED1, and of Ia expressing cells (OX6) in the synovial lining. Intermediate staining with ED2 and of Ia expressing cells in synovial tissue was present. Verschure *et al.* (29) in an immunohistological study on antigen-induced arthritis, induced by intraarticular injection of methylated bovine serum albumin in the knee joints of Wistar rats after systemic preimmunization with this antigen, described a staining pattern in control rats that on some points differed from the results in our control group: ED1 positive staining of the lining was stronger, while Ia expression and ED2 positive staining in the synovial tissue was absent. The Ia expression that we consistently found in synovial tissue of all 4 control rats has also been described in control rats by Holmdahl *et al.* (30) in their study on collagen type II-induced arthritis in Lewis rats. The difference in Ia expression might be explained as a difference between rat strains (Lewis versus Wistar).

Several authors studied the immunohistologic appearance of human control joints. Type I cells, defined as macrophage-antigen positive and DR positive cells (31) make up about 30% of the synovial lining (32,33). This finding correlates well with the occurrence of ED2 positivity and Ia expression in synovial lining in the control rats in our study.

In joints of healthy subjects limited numbers of T lymphocytes are present, mostly perivascularly situated (34,35). B lymphocytes are seldom or never found in synovium of human control joints (35). In control joints of rats we found numbers of T and B lymphocytes comparable with the numbers in human synovium, but the perivascular

distribution pattern found for T cells in humans was absent in rats.

During the acute phase of arthritis we saw an increase in CD4 positive T lymphocytes, ED1 positive cells and ED3 positive cells in the synovium. The number of ED2 positive cells in the synovial lining was decreased compared with control rats. Essentially the same shifts in staining pattern were seen by Verschure *et al.* (29) and also Holmdahl *et al.* (30) described an increase in CD4 positive T cells.

At day 12 both T lymphocytes and macrophages are sparsely present in the synovium. Only at this timepoint CD8 positive cells were present, while CD4 positive cells were not found. This may reflect the partial remission in arthritis between the acute and the chronic phase, that is often found in our model.

The maximal immunohistochemical abnormalities were found at day 33. At this timepoint the most T cells and ED3 positive cells were present. A predominance of CD4 positive T cells, without perivascular clustering was found. This is in accordance with the absence of a vasculitis in our model (15). Allen *et al.* (14) described an occurrence of mainly T helper cells 42 days after injection of streptococcal cell wall fragments; in this model a clustering of T cells around vessels was present.

At later stages of the chronic phase immunohistology shows a partial return to normal, although macroscopically and histologically the arthritis is still active (15). T cells have considerably diminished, ED3 positive cells have disappeared and Ia expression is comparable with control rats. ED1 positive cells, however, are still present in synovial tissue and ED2 positive cells are still diminished in synovial lining compared with control rats. Holmdahl *et al.* (30) also reported a normalization of immunohistological parameters in joints 22 and 90 days after induction of arthritis, while macroscopically the joint inflammation persisted.

Numerous reports describe the immunohistological findings in human rheumatoid arthritis. Although the duration of the disease is not always known, mostly the arthritis has been present for a longer time (1-15 years) in these studies (34,36,37). DR positivity in the synovial lining is increased (34,35,38,39) based on the presence of type I and type II (macrophage-antigen negative, DR positive) synoviocytes (31,32,40). An increased Ia expression in synovial lining is not found in arthritic rats in our study. In the acute phase even a decrease in Ia expression is found. Also the number of ED2 positive cells is decreased.

In the synovial tissue of rheumatoid arthritis patients T lymphocytes are always present, with a T helper to T suppressor/cytotoxic ratio up to 14:1 (38,41). The subsets show a different distribution pattern. T helper cells are mostly found around vessels (34,41), together with DR positive cells (36,37,41) that are probably interdigitating cells (42). T suppressor/cytotoxic cells are found more dispersed in the synovial tissue (34,37,41). Although in our rat study we do not find the same distribution patterns, the predominance of CD4 positive over CD8 positive cells is clearly present.

Several authors describe the presence of B lymphocytes or plasma cells in synovial

tissue (41,43). They are mostly present in follicle-like structures together with T helper cells and interdigitating cells (38,43). This characteristic is a clear distinction from our rat model of arthritis, where only sporadically granuloma-like structures without any B lymphocytes are found.

T cells have been shown to play a dominant role in the development of chronic arthritis in several models, as has been demonstrated by T cell transfer experiments (44), arthritogenicity of T cell lines (45-48) and inhibition of arthritis after depletion of T cells with monoclonal antibodies (49-51). In this study, however, we find T cells predominantly in the early chronic phase; only limited numbers of T lymphocytes are present at later stages of the inflammation. Similarly Noble *et al.* (52) described a temporary increase in number of T cells at the site of inflammation in experimental proliferative immune complex glomerulonephritis. It could be that after initialization of the inflammation only a small number of specific T cells is needed to keep the proces going. In this respect it has been reported that the presence of only one antigen-specific T lymphocyte is sufficient to elicit a delayed type hypersensitivity reaction in mice (53).

ED3 positivity parallels the presence of T cells. This could be the result of T cell-produced cytokines (not being interferon γ or interleukin 2) that are shown to be able to induce ED3 expression *in vitro* (54). The role of these ED3 positive macrophages is not clear. Normally they are only present in lymphoid organs, but in several models of autoimmune diseases, e.g. experimental allergic encephalomyelitis (55) and experimental proliferative immune complex glomerulonephritis (52), they are found at the site of inflammation. In our model they were present in the early chronic phase of arthritis and in the granuloma-like structures at later stages of the disease.

The presence of ED1 positive macrophages in the synovial and periarticular tissue that persisted until (at least) day 90, indicates an important role for these macrophages in the perpetuation of the chronic phase of the disease. In the experimental glomerulonephritis model an important role for ED1 positive macrophages in the development of the disease was suggested (52). In the experimental encephalomyelitis model it was shown that without the presence of macrophages no disease could develop (56). This means that a prominent role for macrophages must be suspected.

In conclusion our results suggest that apart from T helper lymphocytes also macrophages play an important role in the development of experimental arthritis. In the early chronic phase especially ED3 positive macrophages dominate. In the late chronic phase of the disease the role of ED1 positive macrophages probably becomes more prominent. The immunohistological findings in the animal model of arthritis differ from those in rheumatoid arthritis in that a less prominent role for T lymphocytes and MHC class II positive cells during the observation period of 90 days was found.

ACKNOWLEDGMENTS

We thank Mrs I.C.M. Vogels for technical support, Dr. C.D. Dijkstra and Dr. J. Rozing for carefully reading the manuscript, Mr. T.M. van Os for preparing the figures and Mrs. H.J. Elsenbroek-de Jager for secretarial help. This study was supported by a grant from the Nationaal Reumafonds (Dutch Rheumatism Foundation).

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CHAPTER 3

INFLUENCE OF DECONTAMINATION ON INDUCTION OF ARTHRITIS IN LEWIS RATS BY CELL WALL FRAGMENTS OF *EUBACTERIUM AEROFACIENS*. ARTHROPATHIC PROPERTIES OF INDIGENOUS ANAEROBIC BACTERIA*

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* *Ann Rheum Dis, in press 1992.*

SUMMARY

Although the cause (or causes) of rheumatoid arthritis is unknown, many authors have suggested that microorganisms play a part. The intestinal flora in particular has been related to the development of joint inflammation. It has been shown previously that cell wall fragments of several anaerobic Gram-positive intestinal bacteria of human origin are arthritogenic after a single intraperitoneal injection in Lewis rats. The part played by indigenous microflora in this model has now been studied by decontaminating Lewis rats before the injection of *Eubacterium aerofaciens* cell wall fragments. The pattern and severity of arthritis appeared to be comparable in decontaminated and control rats. The second goal of this study was to isolate arthritogenic bacteria from the autochthonous intestinal flora of rats. Only a limited number of bacteria showing a resemblance to arthritogenic strains from human intestinal flora (i.e. *E. aerofaciens* and *Bifidobacterium adolescentis*) could be isolated. These strains did not induce chronic arthritis after intraperitoneal injection. This may explain why spontaneous arthritis did not develop in Lewis rats.

INTRODUCTION

Two models are used to determine the arthritogenicity of bacterial cell wall fragments or purified peptidoglycan-polysaccharide complexes. The intraperitoneal route of injection (IP model, first described for *Streptococcus pyogenes* by Cromartie *et al.*, ref. 1) is used for large bacterial fragments (i.e. $>10^6$ dalton). Subcutaneous injection (SC model, first described for *Mycobacterium tuberculosis* by Pearson, ref. 2) is useful for isolated peptidoglycan or peptidoglycan subunits and larger bacterial fragments. Using the IP model we found that cell wall fragments from *Eubacterium* and *Bifidobacterium* species induced a severe and chronic arthritis in Lewis rats. *Eubacterium* cell wall fragments were also arthropathic in the SC model. *Eubacterium* and *Bifidobacterium* species are Gram-positive major residents of the human intestinal anaerobic flora occurring in numbers exceeding 10^9/gm feces (3-6). These findings add experimental evidence to the hypothesis that the bacterial load in the intestine plays a part in the etiology of rheumatoid arthritis.

Spontaneous arthritis occurred in 0.5% of Sprague Dawley rats in an observation period of six months (7). The incidence increased to 6% after repeated total body irradiation. It is possible that the arthritis was triggered by bacterial products derived from intestinal flora. Irradiation enhances the uptake of whole bacteria (8), but probably also of bacterial products.

Information on the influence of the presence or absence of bacterial flora in arthritis models is limited. Pearson *et al.* (9) showed that adjuvant arthritis could be induced in conventional and in germfree Lobund rats. Others found that germfree Fischer rats were

susceptible to arthritis induction in the adjuvant-induced arthritis model using *M. tuberculosis* (10-12) or the IP model using streptococcal cell wall fragments (13) in contrast to their conventional littermates. Even less is known about the occurrence of arthropathic species among the indigenous bacteria of the intestine of rats. In our previous papers only bacterial strains isolated from human intestinal flora were studied.

In this study the direct influence of autochthonous rat flora on the induction of arthritis by *Eubacterium aerofaciens* cell wall fragments in the IP model was investigated. First, the effect of total intestinal decontamination on the development and severity of cell wall fragments induced arthritis was determined. Second, the intestinal flora of the rat was analyzed for the presence of *Eubacterium* and *Bifidobacterium* species. Cell wall fragments of isolated strains were tested on arthritogenicity.

MATERIALS AND METHODS

Intestinal decontamination

Male Lewis rats (n=10) were decontaminated by adding 1 gram ampicillin, 1 gram neomycin and 1 gram polymyxin B per litre drinking water. Control rats (male, n=5) were given drinking water without antibiotics. Both groups were kept in sterilized cages and fed sterilized food. The composition of the intestinal flora was evaluated by microscopic examination of Gram stained fecal smears and by anaerobic and aerobic culturing of fecal dilutions. Decontamination was checked two weeks after the start of treatment with antibiotics and after six weeks, just before the administration of *E. aerofaciens* cell wall fragments. Antibiotics were continued during the whole experiment. At the end of the experiment decontamination was evaluated once more by fecal smears.

Direct fecal smears

Samples of feces were suspended in saline and spread on an object glass. After Gram staining the samples were examined for bacteria and yeasts. The detection limit of a direct smear was 10^7 bacteria/gm feces wet weight.

Anaerobic culture

Feces of decontaminated and control rats were diluted and plated within one hour. Samples were suspended in the anaerobic diluent with a Stomacher Lab Blender (Colworth, London, UK). Serial ten fold dilutions were prepared from the suspensions.

The anaerobic diluent contained (per litre distilled water): tryptone (Oxoid Ltd, London, UK), 5 gram; glucose, 5 gram; $K_2HPO_4.3H_2O$, 3 gram; KH_2PO_4 0.5 gram; NaCl, 5 gram; L-cysteine hydrochloride (Sigma, St Louis, MO), 0.5 gram; and resazurin (BDH, Poole, UK), 0.002 gram. The pH was adjusted to 7.2. From the dilutions 0.2 ml was plated on anaerobic flasks as described previously (14). A non-selective solid medium for anaerobes (Schaedler broth, Oxoid, with 2% agar, Difco, Detroit, MI) was used. Anaerobic conditions were verified by using the indicator resazurin (0.0002%), which becomes pink at a redox potential greater than -120 mV at pH 7.0 (15). After three days of incubation at 37°C the colonies were counted. The flora was determined according to methods used in previous studies on flora composition (16). The microorganisms were separated into the 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 90% of the cultured organisms could be assigned to one of these groups.

Aerobic counts

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

Isolation and identification of probable arthropathic species

Feces from conventionally housed female Lewis rats (n=2) was diluted and cultured as described under 'Anaerobic culture'. Bacteria which were morphologically assigned as probable *Eubacterium* or *Bifidobacterium* species were subcultured. Pure cultures were identified according to the system of Holdeman *et al.* (17).

The carbohydrate fermentation capacity was determined under anaerobic conditions in peptone yeast extract medium with 0.05% L-cysteine-HCl, 0.0002% resazurin and 0.03% agar in sterilized (121°C, 15 minutes) 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, or xylose. A culture pH <6.0 after five days of incubation was labeled as positive.

The glucose fermentation products were determined by gas chromatography from five day incubates of the strains in peptone yeast broth with 1% glucose at 37°C under anaerobic conditions. (18)

Preparation of bacterial cell wall fragments

E. aerofaciens (ATCC 25986) was cultured overnight at 37°C in Schaedler broth under strictly anaerobic conditions after inoculation with a log phase culture. The strains isolated from rat feces were cultured under the same conditions on Todd Hewitt broth (Oxoid). Bacterial cell wall fragments were prepared as described by Cromartie *et al.* (1) followed by the differential centrifugation procedure of Fox *et al.* (19). Briefly, cells were harvested, washed, and subsequently fragmented with glass beads in a Braun shaker (Melsungen, Germany). Cell walls were collected by 10,000*g* centrifugation, and treated with ribonuclease (Boehringer Mannheim GmbH, Germany) and trypsin (Sigma, USA), washed and sonicated (MSE, Crawley, UK) for 75 minutes. After the sedimentation of debris, the sonicated cell wall suspension was centrifuged at 10,000*g* for 30 minutes; the 10,000*g* supernatant was centrifuged twice at 100,000*g* for 60 minutes. Both 100,000*g* pellets were collected, resuspended in phosphate buffered saline and used for intraperitoneal 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 (20) and Dische and Shettles (21) respectively. The total amount of carbohydrates was determined according to Dubois *et al.* (22) using galactose as standard.

Induction of arthritis

Six weeks after start of the decontamination the decontaminated and control rats received an intraperitoneal injected of an aqueous suspension of *E. aerofaciens* cell wall fragments. Rats weighed 250-330 gram at the time of injection.

Groups of five conventionally housed female Lewis rats (Harlan Sprague Dawley, Bicester, UK), weighing 110-185 gram received an intraperitoneal injection of cell wall fragments of the bacteria isolated from rat feces. All rats received a cell wall dose equivalent to 25 μg of muramic acid per gram of body weight. The rats were observed for the development of paw inflammation at regular intervals during 60 days; the diameters of their wrists and ankles were measured with a vernier caliper at the distal end of the radius and at the malleoli respectively.

Statistical analysis

The acute and chronic phase of the arthritis in decontaminated and control rats were compared as follows: for each rat in the decontaminated and control groups the mean increase in the sum of the paw diameters in the acute (day 1-15) and chronic (day 19-60) phases was calculated using the sum of the paw diameters of the same rat at day 0 as reference. The values for each phase found in the two groups were compared using the Mann Whitney U test.

Histology

After 60 days, rats were killed by cardiac puncture bleeding under ether anaesthesia. Skinned paw joint specimens were fixed in 4% formalin, decalcified in 5% (v/v) formic acid for five days, and embedded in paraffin. Liver specimens were fixed in Bouin's fluid before embedding in paraffin. Sections were stained with hematoxylin and eosin.

RESULTS

Evaluation of intestinal decontamination

Gram stained fecal smears showed that the intestinal flora of decontaminated rats was significantly reduced after 14 days on drinking water with antibiotics. Culturing of feces of intestinally decontaminated rats showed that anaerobic bacteria were no longer detectable (less than 10^3/gm feces wet weight) and aerobes had decreased considerably (median of $1.7x10^4$, range $<10^3$-$1.1x10^6$ bacteria/gm feces). In control rats $4.8x10^{10}$ (range $3.5x10^{10}$-$6.2x10^{10}$) anaerobes and $9.7x10^7$ (range $1.2x10^7$-$1.8x10^8$) aerobes/gm feces wet weight were found. After six weeks of decontamination five rats were selected for induction of arthritis, based on bacterial counts below detection level of culturing (10^3/gm feces). At the end of the experiment no or only small amounts of bacteria could be found in fecal smears of these rats. All five rats had yeast in their fecal smears. At the time of death decontaminated rats had distended ceca filled with a watery content, a phenomenon characteristic of germfree rodents (9,23).

The composition of the flora of the control rats did not change during the experiment.

Induction of arthritis with and without intestinal decontamination

After six weeks of intestinal decontamination the effect on arthritis induction by cell

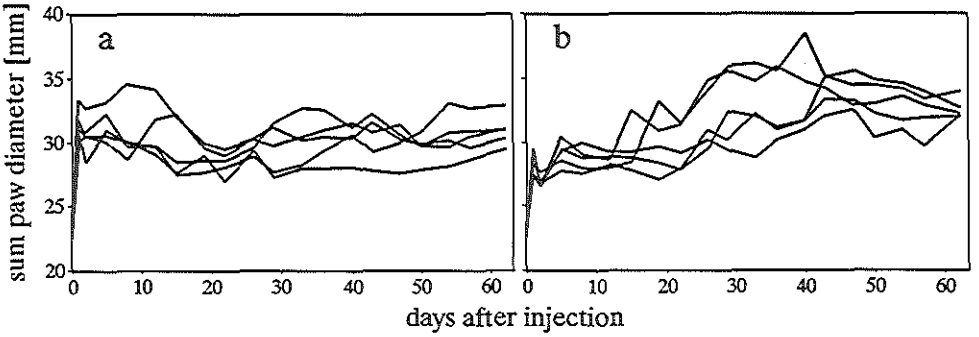

Figure 1. Induction of arthritis by *E. aerofaciens* cell wall fragments (25 µg MA/gm body weight) in male Lewis rats. (a) control rats, (b) decontaminated rats.

wall fragments of *E. aerofaciens* was tested. The results show that control rats with a normal flora (figure 1a) and decontaminated rats (figure 1b) developed an acute chronic arthritis. Neither for the acute nor the chronic phase were statistically significant differences found between the two groups. The chronic arthritis was confirmed by histology at the end of the experiment. Figure 2a shows a severely damaged joint with infiltration of the joint cavity with polymorphonuclear cells and erosion of the cartilage and bone. Three rats (one of the control rats and two of the decontaminated rats) had macroscopic liver granulomas. Histologically the inflammation appeared to be localized mainly around the central veins and in the portal tracts, and consisted of a predominantly histiocytic infiltrate with sporadic lymphocytes (figure 2b).

Presence of potentially arthropathic bacteria in the rat flora

After culturing of feces of a conventional Lewis rat the flora was analyzed in detail. Thirty five colonies of bacteria morphologically belonging to *Eubacterium*, *Bifidobacterium*, or related genera were further subcultured. A number of the pure cultures were similar, so 19 different strains were identified. Only one of the strains could be assigned to the genus *Eubacterium*, none belonged to the genus *Bifidobacterium*. The strains belonged to the genera *Peptostreptococcus* (species: *intermedius*, *productus*, *anaerobius*, *saccharolyticus*), *Peptococcus* (species: *magnus*, *constellatus*), *Eubacterium* (species: *lentum*) and *Ruminococcus* (species: *albus*).

On basis of morphology and the production of volatile acids and ethanol, strain R11 (*P. saccharolyticus*), R12 (*P. productus*) and R19 (*E. lentum*) showed the closest resemblance to the arthropathic *E. aerofaciens*.

Arthritis induction by cell wall fragments from three rat strains of bacteria

Cell wall fragments from the rat strains *P. saccharolyticus*, *P. productus*, and *E. lentum* were tested for the induction of arthritis. Table 1 gives the muramic acid, rhamnose, and total carbohydrate contents of the cell wall fragments of the three rat strains compared with *E. aerofaciens* cell wall fragments. Figure 3 shows the results of the induction of arthritis; the cell wall fragments of the three rat strains of bacteria induced a self-limiting acute joint inflammation. Histological examination 60 days after the injection of cell wall fragments showed no signs of active or previous inflammation.

DISCUSSION

The supply of ampicillin, a moderately absorbable antibiotic, by mouth, in combination with neomycin and polymyxin B both non-absorbable, almost completely eliminated the intestinal flora of rats. After six weeks of intestinal decontamination, predominantly yeasts

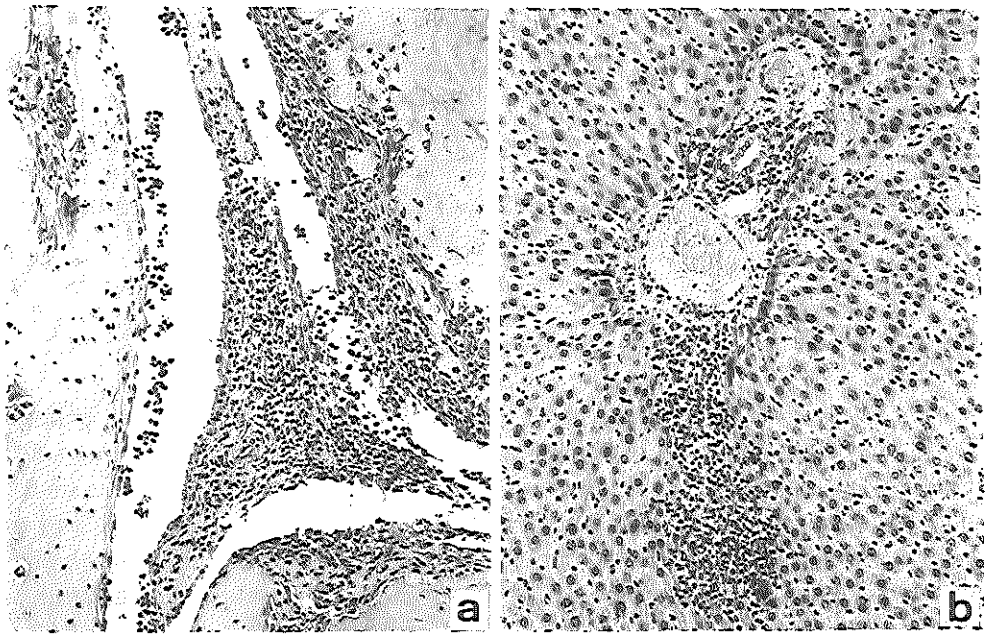

Figure 2. Histology of arthritic rat 60 days after injection with cell wall fragments of *E. aerofaciens*. (a) section of an inflamed hind paw, with marginal erosions of bone (left side), severely damaged cartilage (bottom right) and polymorphonuclear infiltrate in the joint space, (b) section of a granulomatous liver, portal tract with histiocytic infiltrate extending into the parenchyme (hematoxylin and eosin staining, 120 x).

Table 1. Chemical composition of cell wall fragments used for induction of arthritis. Contents are expressed as percentage of dry weight.

	Cell wall fragments*			
	EA	PS	PP	EL
Muramic acid	15.7	20.4	16.2	17.5
Rhamnose	23.4	41.3	30.0	43.6
Total carbohydrates	64.4	74.3	60.1	76.3

* Cell wall fragments from *E. aerofaciens* (EA) and from rat strains *P. saccharolyticus* (PS), *P. productus* (PP) and *E. lentum* (EL).

and, in some rats, a limited number of Gram-negative rods were present. These persisted until the end of the experiment. The occurrence of yeasts after intestinal decontamination has been observed before (24). Some features of germfree rats, such as mild diarrhoea, enlarged cecum (9,23) and the absence of intestinal flora derived enzymes, have also been described for intestinally decontaminated rats (24-26). We also found diarrhoea and cecal distension in decontaminated rats.

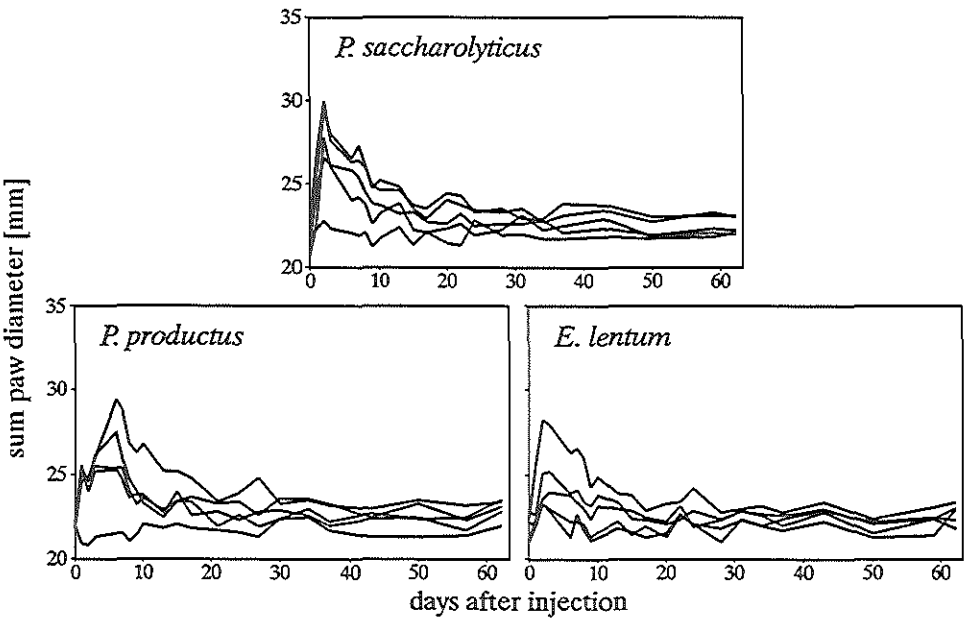

Figure 3. Induction of arthritis by cell wall fragments (25 μg MA/gm body weight) from three strains isolated from rat feces.

Elimination of the largest pool of bacterial antigens did not alter the susceptibility for arthritis induced by *E. aerofaciens* cell wall fragments. Conventional and intestinally decontaminated rats developed a comparable acute and chronic arthritis. Neither the severity nor the pattern of arthritis was influenced by the presence or absence of the indigenous flora. Apparently the amount of cell wall fragments (approximately 20 mg/rat) injected contained sufficient peptidoglycan to start and maintain a joint inflammation for at least 60 days. These findings are consistent with the results of Pearson *et al.* (9) who found in the adjuvant-induced arthritis model that germfree Lobund rats were as susceptible to the induction of arthritis by *M. tuberculosis* as conventional animals, although the onset of arthritis was slightly delayed. In these experiments an even smaller amount of bacterial material was sufficient (0.5 mg/rat). An influx of peptidoglycan from the gastrointestinal tract is obviously not necessary for development or continuation of arthritis.

Others have also found that the germfree status does not prevent arthritis. On the contrary, rat strains normally not or hardly susceptible developed more often and more severe arthritis when they were germfree. This is shown both in the adjuvant-induced arthritis model (10) and in the streptococcal cell wall-induced arthritis model (13). The experiments of Kohashi *et al.* (11,12) indicate a protective role for Gram-negative bacteria: *Escherichia coli* or *Bacteroides* monocontaminated Fischer rats developed a less severe arthritis than germfree Fischer rats. Gram-positive *Lactobacilli* increased the susceptibility of arthritis of germfree Fischer rats. This effect was abolished by simultaneous administration of *E. coli*. The lipopolysaccharides of Gram-negative bacteria is said to protect against arthritis (27), whereas bacterial peptidoglycan is capable of inducing or aggravating the disease. The protective effect of the intestinal flora in rats which are not susceptible to arthritis has until now only been investigated in selectively contaminated germfree rats. It would be interesting to observe the effect of intestinal decontamination on induction of arthritis in nonsusceptible rats. A continuing lack of susceptibility after intestinal decontamination might mean that rats become tolerant to arthritis by their intestinal flora in the neonatal state.

It is remarkable that we never observed a spontaneous arthritis in Lewis rats, although they are very susceptible to the induction of arthritis both in the adjuvant-induced arthritis model and in the intraperitoneal model. This is in contrast with the study of Trentham *et al.* (7), who described a spontaneous development of arthritis in 0.5% of female Sprague-Dawley rats. The incidence increased to 6% after total body irradiation with 17 fractions of 2Gy. Translocation of bacteria from the intestine to the mesenterial lymph nodes has been described after irradiation for mice (8), and after thermal injury (28) or hemorrhagic shock (29) for rats. Without the presence of arthritogenic bacteria in the intestine, however, it is unlikely that arthritis will occur in either conventional or irradiated animals.

In our previous studies we have shown the arthritogenicity of several anaerobic

bacteria of the human intestinal flora in Lewis rats. *Eubacterium* and *Bifidobacterium* species especially were able to induce a severe chronic arthritis. We have tried to isolate potentially arthritogenic bacteria (i.e. *Eubacterium* and *Bifidobacterium* species) from the indigenous flora of Lewis rats by a method used for the isolation of arthropathic *E. aerofaciens* strains from the intestinal flora of healthy subjects and rheumatoid arthritis patients. Although 19 strains resembled *Eubacterium* or *Bifidobacterium* species morphologically, however, only one of them could be identified as belonging to the genus *Eubacterium* (*E. lentum*). This result is in agreement with descriptions of the intestinal flora of conventional rats by several authors. Although Raibaud *et al.* (30) found *Bifidobacterium* and *Eubacterium* species among the dominant intestinal flora of conventional rats occurring in numbers between 10^7 and 10^{10}, more recent studies give lower numbers of these genera. Rowland (31) found less than 0.8% bifidobacterium in the feces of Sprague Dawley rats, no *Eubacterium* species were isolated. In ceca of Wistar rats, Morishita and Miyaki (32) found a maximum of 10^6 *Bifidobacterium* at the age of four to nine weeks. Younger and older rats had lower numbers of *Bifidobacterium*. In this study *Eubacteria* were not considered as a separate group, but were counted as *Catenabacteria* together with anaerobic *Lactobacilli*, preventing any conclusions about the numbers of this genus.

In addition to *E. lentum*, two more strains isolated from rat flora were selected for induction of arthritis. Although we have chosen the strains that most resembled the arthropathic *E. aerofaciens*, none of them was able to induce a chronic arthritis. All three strains gave only an acute arthritis. This has been described before for *P. productus*, but *E. lentum* was capable of inducing a chronic arthritis after the initial acute joint inflammation in earlier experiments, using ATCC strains (5).

The failure to induce arthritis cannot be explained by a low muramic acid content because this was as high as in *E. aerofaciens* cell wall fragments. Nor can it be explained by the rhamnose content, which was even higher than in *E. aerofaciens* cell wall fragments. Other, unknown, characteristics are to be held responsible for the difference in arthritogenicity between *E. aerofaciens* and the three rat strains of bacteria.

Although we have tested only a limited number of bacterial strains from the rat flora, we believe that there is no abundant amount of arthropathic bacteria in the intestinal tract of Lewis rats. This absence of triggering peptidoglycan may explain why we never found a spontaneous arthritis in susceptible Lewis rats, although this rat strain is extremely susceptible to the induction of arthritis by cell wall fragments of intestinal anaerobic bacteria of human origin.

ACKNOWLEDGMENTS

We thank Mr. T.M. van Os for his skillful photographic assistance and Mrs. H.J. Elsenbroek-de Jager for

secretarial help. This study was financially supported by the Nationaal Reumafonds (Dutch Rheumatism Foundation).

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CHAPTER 4

ARTHRITOGENICITY OF *EUBACTERIUM* SPECIES IN THE ADJUVANT ARTHRITIS MODEL*

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* *Published as a Letter in: J Rheumatol, in press 1992.*

SUMMARY

To investigate the arthritogenicity of cell wall fragments of intestinal anaerobic bacteria (especially *Eubacterium* species) we previously used a model in which cell wall fragments were injected intraperitoneally in Lewis rats. This model, however, has two disadvantages: 1) large quantities of material are needed, 2) small fragments can not be tested in this model. Because in adjuvant-induced arthritis (induced by injecting Freund's complete adjuvant subcutaneously) these disadvantages are absent, we were interested in comparing both models. It appeared that cell wall fragments of some *Eubacterium* species were arthritogenic after subcutaneous injection, but not after intraperitoneal injection and vice versa. The possible origin of these differences is discussed.

INTRODUCTION

Several models are used to investigate rheumatoid arthritis in rats. In two models bacteria or bacterial cell wall fragments are used as inducers of arthritis. The first model is the streptococcal cell wall induced arthritis, first described by Cromartie *et al.* (1). In this model an intraperitoneal injection of cell wall fragments suspended in phosphate buffered saline causes an acute arthritis followed by a chronic arthritis (IP model). The second model is the adjuvant-induced arthritis, first described by Pearson (2), in which killed *Mycobacterium tuberculosis* cells are suspended in Freund's incomplete adjuvant and injected subcutaneously (SC model). After two weeks a chronic arthritis develops.

We have used the IP model in our studies to investigate the role of anaerobic Gram-positive bacteria of the human intestinal flora in arthritis (3-6). Several bacteria (e.g. *Eubacterium aerofaciens* and *Bifidobacterium adolescentis*) were very potent in giving an arthritis in this model (3,4). Between genera but also within one genus (*Eubacterium*) large differences in arthritogenicity occurred (5). Within a species however, e.g. *Eubacterium aerofaciens*, the arthropathic properties varied only a little (6).

The SC model has two advantages over the IP model. First in the SC model less bacterial material is needed to induce arthritis. Secondly the arthropathic properties of smaller molecules (i.e. $<5x10^7$ dalton) can be tested in the SC model, but not in the IP model (7). We therefore wanted to use the SC model for future research. In this study we investigated whether there were significant differences in arthropathic properties of *Eubacterium* species in the SC model compared with the IP model.

MATERIALS AND METHODS

Bacteria

Eubacterium aerofaciens ATCC 25986, *Eubacterium contortum* ATCC 25540, *Eubacterium lentum* ATCC 25559, *Eubacterium tortuosum* ATCC 25548 and *Eubacterium limosum* ATCC 8486 were obtained from the American Type Culture Collection (Rockville, MD); *Eubacterium rectale* was isolated from the fecal flora of a healthy subject and was identified according to Holdeman *et al.* (8). *Streptococcus pyogenes* was obtained from the bacteriological laboratory, Academic Hospital Rotterdam, Dijkzigt. *Streptococcus pyogenes* was cultured overnight at 37°C on Todd Hewitt broth (Oxoid Ltd, London, UK). *Eubacterium* species were cultured overnight at 37°C in Schaedler broth (Oxoid) under strict anaerobic conditions. *Mycobacterium tuberculosis* (H37RA) was obtained from Difco (Detroit, MI).

Preparation of cell wall fragments

Bacterial cell wall fragments (CWF) were prepared as described before (6). In short, cells were disrupted by shaking with glass beads. After incubation with ribonuclease and trypsin the cell walls were sonicated for 75 minutes and CWF were harvested by differential centrifugation at 10,000*g* and 100,000*g*. The 100,000*g* pellet was used in the experiments.

Animals and induction of arthritis

Female Lewis rats (Harlan Sprague Dawley, Bicester, Oxfordshire, UK) weighing 158-243 gram were used. Groups of 5 rats were injected subcutaneously in the base of the tail with 1 mg of CWF suspended in 100 μl of Freund's incomplete adjuvant (Difco). One group was injected with Freund's complete adjuvant: *Mycobacterium tuberculosis* whole cells suspended in Freund's incomplete adjuvant. Rats were observed for development of arthritis for at least 100 days. Paw diameters were measured at malleoli and distal end of the radius with a vernier caliper twice a week until day 50 and once a week till the end of the experiment.

Chemical analysis

Muramic acid and rhamnose contents were determined as described by Hadzija (9) and Dische and Shettles (10).

Table 1. Arthritogenicity of bacterial strains.

Bacterial strain	arthritis pattern*			nr. arthritis /nr. injected
	ankle/wrist	small joints	tail	
M. tuberculosis				
whole cells	4	1	1	4/5
CWF	4	2	1	4/5
S. pyogenes	2	1	1	3/5
*E. aerofaciens 1***	2	0	2	2/5
*E. aerofaciens 2***	5	0	4	5/7
E. limosum	0	0	0	0/5
E. tortuosum	1	0	0	1/5
E. lentum	2	1	1	2/5
E. contortum	1	1	0	1/5
E. rectale	4	1	2	4/5

* Number of rats with inflammation at the mentioned sites.

** Two seperately prepared batches of cell wall fragments from the same strain of *E. aerofaciens*.

Histology

At the end of the experiment rats were sacrificed by cardiac puncture bleeding under ether anesthesia. Skinned paw specimens were fixed in 4% buffered paraformaldehyde solution, decalcified in 5% formic acid and embedded in paraffin. Seven μm sections were stained with hematoxylin and eosin.

RESULTS

Arthritis induction by cell wall fragments

Table 1 lists the results with the different bacterial strains. *Mycobacterium tuberculosis* was not only arthritogenic when injected as whole cells, but also when injected as cell wall fragments. Also *Streptococcus pyogenes* CWF injected with Freund's incomplete adjuvant induced joint inflammation.

There was a large difference in arthritogenicity between the several *Eubacterium* species. *E. aerofaciens* and *E. rectale* were very potent in inducing arthritis. *E. limosum* on the contrary failed to give any inflammatory symptoms. Also within a species differences were found: for example not all rats injected with *E. aerofaciens* developed arthritis and the time between injection and occurrence of arthritis varied considerably (figure 1). Once the joint inflammation had developed it lasted till the end of the experiment with some fluctuation in the severity. Most arthritogenic strains gave not only symptoms at the

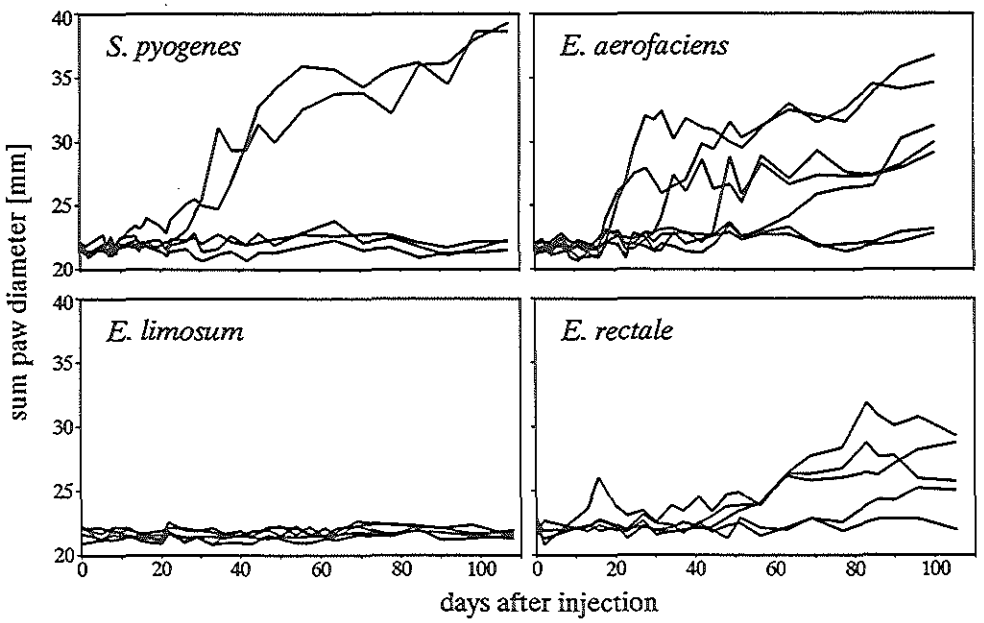

Figure 1. Arthritis induction by cell wall fragments from Gram-positive bacterial strains.

ankles or wrists but also at the smaller joints of the paws and at the tail (table 1). Control rats injected with Freund's incomplete adjuvant showed no signs of inflammation.

Chemical analysis

Table 2 shows the muramic acid and rhamnose contents of the several CWF. Muramic acid contents varied within a limited range except for CWF from *M. tuberculosis* which showed a very low muramic acid content. Rhamnose contents showed much more dispersion with a maximum of 59% for *E. limosum* and a minimum of 1% for *M. tuberculosis* CWF.

Histology

All rats that showed inflammatory symptoms also showed histological signs of arthritis. Paw sections of a rat injected with *M. tuberculosis* whole cells showed marginal erosions of the cartilage and invasion of the bone by granulomatous tissue consisting of histiocytes

Table 2. Chemical composition of cell wall fragments*

Bacterial strain	Muramic acid	Rhamnose
M. tuberculosis	5	1
S. pyogenes	20	45
*E. aerofaciens 1***	16	25
*E. aerofaciens 2***	18	23
E. limosum	20	59
E. tortuosum	14	5
E. lentum	15	36
E. contortum	18	31
E. rectale	19	2

* Expressed as percentage of dry weight.

** Two seperately prepared batches of cell wall fragments from the same strain of *E. aerofaciens*.

(large cells with pale nuclei) and lymphocytes. Sometimes multinucleated osteoclasts were found. Synovial tissue was fibrous with predominantly fibroblasts, histiocytes and lymphocytes. The synovial lining appeared normal, but in the joint space numerous polymorphonuclear (PMN) cells were found. Muscle and tendon sheath were infiltrated by histiocytes.

The characteristics found with paw sections of a rat injected with *M. tuberculosis* CWF differed somewhat from those of *M. tuberculosis* whole cells. Although both showed bone and cartilage erosions, only in *M. tuberculosis* whole cells the bone marrow was infiltrated by granulomatous tissue. In the bonemarrow of a *M. tuberculosis* CWF rat no infiltration by granulomatous tissue but an enhanced hemopoiesis was seen. Also there was less fibrosis of the synovial tissue and no inflammation of muscle was present compared to *M. tuberculosis* whole cells injected rats. In the synovial tissue many PMN cells were found, indicating that the proces was still very active at the time of sacrifice. A characteristic not encountered in *M. tuberculosis* whole cells sections but present in *M. tuberculosis* CWF sections was the apposition of new bone.

Sections of a rat injected with *S. pyogenes* (figure 2a) showed grossly the same characteristics as those of *M. tuberculosis* whole cells. However, no fibrosis of synovial tissue was found and bone apposition occurred as in *M. tuberculosis* CWF sections.

Sections of a rat injected with *E. aerofaciens* (figure 2b) showed severe damage of the cartilage and bone by granulomatous tissue with histiocytes and some PMN cells. Along the eroded cartilage chondroclasts are found. Bone apposition was often encountered. Synovial tissue contained many histiocytes, some lymphocytes and some PMN cells; occasionally fibrosis was present. In the joint space PMN cells were seen and in muscle a lymphocytic infiltrate was sometimes found.

In the sections of an *E. rectale* injected rat similar but more severe inflammatory

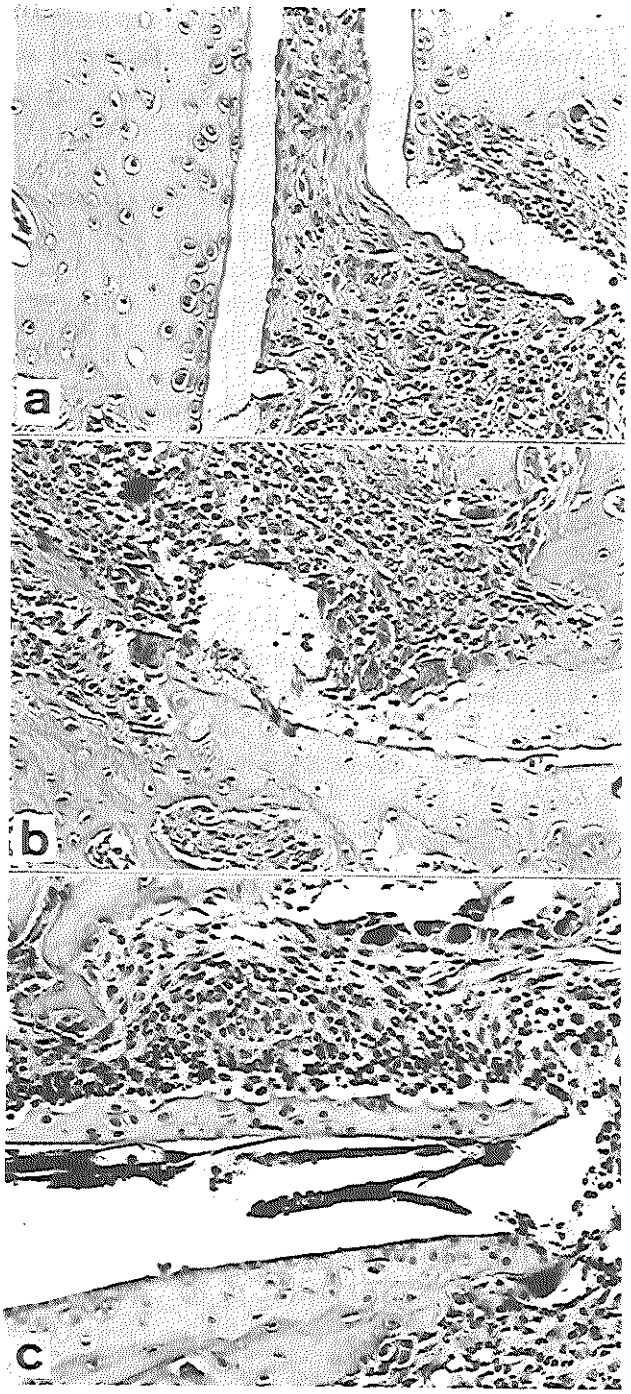

Figure 2. Histology of arthritic hind paws (magnification: 180 x).

a. Induced by cell wall fragments of *S. pyogenes*. Marginal erosion of bone and cartilage is seen. Hyperplastic synovium extending into the joint cavity with an infiltrate of lymphocytes, polymorphonuclear cells and histiocytes is visible.

b. Induced by *E. aerofaciens* cell wall fragments. Marginal erosions and infiltration of bone marrow with granulomatous tissue is seen. Synovium is infiltrated by mononuclear and polymorphonuclear cells. Along the eroded cartilage a number of giant cells are visible.

c. Induced by *E. rectale* cell wall fragments. Severe destruction of bone is present with loose remnants of cartilage. Eroding tissue contains many polymorphonuclear cells, histiocytes, some lymphocytes and some multinucleated giant cells.

characteristics with a more active component were present (figure 2c). Sections of *E. lentum* and *E. tortuosum* injected rats were the only ones where synovial lining hyperplasia was found. They also showed bone and cartilage erosions by granulomatous tissue and bone apposition. Synovial tissue as well as tendons were infiltrated by histiocytes. The joint cavity was filled with PMN cells.

The *E. contortum* injected rat that developed a mild arthritis showed histologically also mild inflammatory symptoms: minor marginal erosions, no granulomatous infiltration of bone marrow or bone apposition, mild histiocytic infiltrate of muscle and synovial tissue and no exudation of inflammatory cells into the joint space. Sections of the inflamed tail showed more severe bone erosions and infiltrations of periarticular tissue and muscles by histiocytes. Also bone apposition could be found.

No histological abnormalities were found in sections of a rat injected with *E. limosum* or control rats.

DISCUSSION

The aim of this study was to compare the SC model with the IP model. In the SC model mainly *M. tuberculosis* whole cells have been used for arthritis induction, although two studies concerned CWF of *M. tuberculosis* (11,12). In these studies as well as in our present study CWF of *M. tuberculosis* proved to be arthritogenic in the SC model. Recently a report has been published (13) in which was demonstrated that *M. tuberculosis* whole cells are also arthritogenic in the IP model, however, a severe chemically induced peritonitis prior to ip injection was necessary to obtain this result.

Also *S. pyogenes* CWF, predominantly used in the IP model, were arthritogenic in the SC model (14, this report). The incidence of *S. pyogenes* induced arthritis in the SC model, however, is lower than in the IP model (40% and 100% respectively). The same was found for *E. aerofaciens* induced arthritis with incidences of 56% and 100% respectively. Also the onset of arthritis differed between the two models. In the IP model less variation occurred (onset of chronic arthritis directly after the acute phase, 2-3 weeks after injection, ref. 1,4,5), while in the SC model arthritis sometimes developed even after 40-50 days. No acute arthritis starting within a week after injection, was found in the SC model (nor has it ever been described). These differences might be explained in the following way: In the IP model a high dose (±20 mg) of bacterial material is injected in phosphate buffered saline, which makes it readily available for distribution over the body. In the SC model the CWF are injected suspended in oil, which will delay the distribution. The lower dose that is used (1 mg) may explain the lower incidence, the delay in distribution may explain the late onset. Kohashi *et al.* (14) also found low incidences of *S. pyogenes* induced arthrits (30%), but they found no delay in onset (2-3 weeks). This may be due to injection in inguinal lymph nodes instead of subcutaneously. Besides the

incidence and the onset of arthritis also the sites where the inflammation occurs differs somewhat between the two models. In the IP model the arthritis is almost exclusively localised in the wrists and ankles, whereas in the SC model inflammation of the smaller joints and the tail was more frequently encountered.

Although several species within the genus *Eubacterium* induced chronic arthritis in both models (eg. *E. aerofaciens*, *E. lentum*, *E. contortum*) others gave strikingly different results. *E. limosum* CWF gave only an acute arthritis in the IP model and failed to give any inflammatory symptoms in the SC model. This is easy to understand, because in the SC model an acute arthritis never occurs. Furthermore it has been described that *E. limosum* has no adjuvanticity (15), which is a prerequisite for arthritogenicity in the SC model according to Kohashi *et al.* (16).

It is less comprehensible why *E. tortuosum* CWF, that also gave only an acute arthritis in the IP model, gave a chronic arthritis in the SC model, albeit in only one rat. And even more astonishing is it that *E. rectale* CWF induced a severe chronic arthritis in the SC model, while they failed to give any inflammatory symptoms in the IP model. A possible explanation could be that both these cell walls contain too little of a "protecting factor" against degradation to be arthritogenic in the IP model, but because of the injection of CWF suspended in oil this factor is not needed in the SC model. Rhamnose has been proposed to play such a role in the IP model (17,18) although we could not support this hypothesis with our earlier results (5,6). However it is striking that CWF from both species have a low rhamnose content.

Histologically the arthritis is grossly the same in both models. In the IP model (5) as well as the SC model (19,20, this report) the main characteristics of the joint inflammation are erosion, starting at the cartilage-synovial junction; infiltration of synovial tissue by lymphocytes, PMN cells and histiocytes; exudation into the joint cavity of predominantly PMN cells; fibrosis of synovial tissue and bone apposition. The histopathological findings described for RA in humans include synovial lining hyperplasia; infiltration of synovial tissue by lymphocytes, plasmacells, macrophages and dendritic cells; exudation into the joint cavity of PMN cells; erosions at the cartilage-synovial junction. In later phases more fibrosis, ankylosis and osteoporosis is seen (21-24). The patterns of arthritis in rat models and human RA thus have much in common, the two major dissimilarities being the occurrence of plasma cells and osteoporosis in humans and the more often encountered bone apposition in rats.

This study shows that in both the IP and the SC model CWF can be arthritogenic. But it depends on the strain from which the CWF are derived whether they induce arthritis in both or only one of the two models. The arthritogenicity probably is determined by the route of injection, the vehicle in which the CWF are suspended and the chemical (and antigenic) composition of the CWF. This means that both models can be used to investigate the arthropathic properties of CWF, although non-arthritogenicity of the injected material in one model does not mean that it lacks arthritis inducing capacities

completely. The IP model in our opinion comes closer to the hypothetical model as described by Bennett (25, soluble bacterial fragments passing the bowel wall, distributing over the body and causing local inflammatory symptoms, especially in the joints). The CWF are suspended in a watery solution and injected ip, close to the gastrointestinal tract.

However, a sometimes important disadvantage is the large amount of CWF that is needed to induce arthritis. This disadvantage is absent in the SC model. Another advantage of the SC model is the possibility to investigate smaller sized particles. As shown by Fox *et al.* (7) CWF smaller than $5x10^7$ dalton give only acute inflammatory symptoms in the IP model. As we are more interested in chronic joint inflammation, the IP model is inadequate for investigating smaller fragments, like soluble peptidoglycan-polysaccharide complexes that can be isolated from feces (results to be published). The SC model however offers a good alternative in such a situation.

ACKNOWLEDGMENT

We thank Mr. T.M. van Os for skillful photographic assistence and Mrs. H.J. Elsenbroek-de Jager for secretarial help. This study was supported by the Nationaal Reumafonds (Dutch Rheumatism Foundation).

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CHAPTER 5

INDUCTION OF ARTHRITIS IN RATS BY SOLUBLE PEPTIDOGLYCAN-POLYSACCHARIDE COMPLEXES PRODUCED BY HUMAN INTESTINAL FLORA*

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* *Arthritis Rheum 1991;34:1611-6.*

SUMMARY

Peptidoglycan-polysaccharide complexes were isolated from feces of a healthy subject and from ileostomy fluid from 5 patients. Peptidoglycan-polysaccharide complexes were tested for arthritogenicity in a rat model, by subcutaneous injection in Freund's incomplete adjuvant. Complexes from the healthy subject did not induce arthritis, but those from ileostomy fluid of 1 of the patients induced severe, chronic joint inflammation. We concluded that peptidoglycan-polysaccharide complexes from intestinal flora are potentially arthritogenic in rats. This arthritogenicity may be influenced by the content of muramic acid and rhamnose in these complexes.

INTRODUCTION

Rheumatoid arthritis (RA) is a disease of unknown etiology and pathogenesis. Several authors have proposed a causal role for intestinal bacteria (1-4). Bacterial debris may pass the bowel wall and either be deposited in the joints and give rise to local inflammation or stimulate the immune system and cause arthritis as one of the immune responses (1,2). Studies from our laboratory revealed that isolated cell wall fragments from anaerobic Gram-positive bacteria present in high numbers (i.e. 10^{10}/gm feces) in the human intestinal flora could induce arthritis in a rat model (5-8). Although the bacteria originated from a natural source (the human bowel) the cell wall fragments were prepared by an artificial procedure using disintegration by glass beads, ultrasonication and enzyme digestion. Earlier studies from our group showed that bacterial fragments were present in feces as soluble peptidoglycan-polysaccharide complexes with a high molecular weight (9). These peptidoglycan-polysaccharide complexes shared antigens with the major components of the anaerobic intestinal flora, including the species with arthritogenic properties (10). Antibodies to peptidoglycan-polysaccharide complexes were found in human sera, showing that these complexes can circulate within the body. In the present study, we investigated the arthritogenic properties of soluble intestinal peptidoglycan-polysaccharide complexes.

PATIENTS AND METHODS

Feces and ileostomy fluid

Feces of 1 healthy subject and ileostomy fluid of 5 patients (ages 37-70 years, median 45 years) were studied. The duration since the performance of the ileostomy ranged from 6 years to 20 years (median 10 years), and all of the patients had optimal function of the

ileostomy at the time of study. The reason for ileostomy was the need for removal of colon and terminal ileum because of Crohn's disease, ulcerative colitis, or perforation. None of the patients was on a restricted diet, and the only drugs used were loperamide, folic acid, or vitamin B. Feces were stored at -20°C, within 2 hours after passage. Ileostomy fluid was collected overnight in a bag and hereafter stored at -20°C until used.

Preparation of peptidoglycan-polysaccharide complexes

Peptidoglycan-polysaccharide-complexes from feces of a healthy subject and ileostomy fluid were prepared as described by Hazenberg *et al.* (9). Briefly, feces or ileostomy fluids were diluted in distilled water (40 gram in 100 ml) and homogenized in a Model 400 Lab Blender (Stomacher, Colworth, UK). After cambric gauze filtration, the suspension was centrifuged for 45 minutes at 5,000*g*. Four volumes of 96% ethanol was added to 1 volume of supernatant, and after 2 hours at 4°C, the precipitate was centrifuged for 15 minutes at 5,000*g*. The pellet was dissolved in distilled water and dialyzed against water for 48 hours. Next, the suspension was centrifuged for 1 hour at 100,000*g* and the clear supernatant (ethanol precipitate) was collected.

Size exclusion chromatography was performed using dilutions of 15-60 mg/30 ml dilutions (depending on the viscosity of the solution), with a TSK HW75 (gel bed 700 x 26 mm; Merck, Darmstadt, Germany) and distilled water with 0.4% sodium azide as eluent. After passage of 100 ml void volume, fractions (8 ml/5 minutes) were collected and assayed for their protein and carbohydrate contents. High molecular weight fractions containing carbohydrates but no proteins were pooled, dialyzed, and lyophilized. In later experiments, the ethanol precipitate from ileostomy fluid from patient 1 was filtrated with a Minitan Ultrafiltration system, using a PTMK filter with a molecular weight limit of 300,000 (Millipore, Bedford, MA) instead of the chromatography step. This resulted in isolation of peptidoglycan-polysaccharide complexes with the same molecular weight range and the same chemical composition as those isolated with chromatography. From 100 gram of feces or ileostomy fluid, about 50 mg peptidoglycan-polysaccharide complexes could be retrieved by this procedure.

Chemical analysis

Total carbohydrate content and rhamnose content were determined as described by Dubois *et al.* (11) and Dische and Shettles (12), respectively. Muramic acid content was determined according to the method of Hadzija (13), with some modifications. Briefly: samples were hydrolyzed 18 hours at 90°C with the same volume of 5M H_2SO_4 and neutralized with 10M NaOH. On hundred microliters of hydrolyzed or nonhydrolyzed

samples was incubated with 50 µl 1M NaOH at 36°C for 30 minutes. One milliliter of 18.8M H_2SO_4 was added, and the samples were heated for 10 minutes at 100°C. After cooling, samples were mixed first with 10 µl of 4% $CuSO_4.5H_2O$ and then with 20 µl of 1.5% *p*-hydroxydiphenyl in ethanol. The blue color development was maximal after 30 minutes at 30°C. Absorbance at 570 nm was determined in a Titertek Multiscan (Flow, Irvine, Scotland). *N*-acetylmuramic acid (A-3007, Sigma, St. Louis, MO) (0-100 µg) was used as a standard. In hydrolyzed samples, the estimated amount of muramic acid was the total amount of muramic acid present. In nonhydrolyzed samples, the amount of muramic acid not bound to peptide side chains was estimated.

The assay described by Bradford (14) was used to determine the protein content of peptidoglycan-polysaccharide complexes. Protein content was expressed as absorbance at 280 nm as measured in chromatography fractions.

Induction of arthritis

Female Lewis rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany) weighing 125-150 gram at the start of the experiment, were used. Rats were injected subcutaneously in the base of the tail with 1 mg of peptidoglycan-polysaccharide complexes ground in 100 µl Freund's incomplete adjuvant (Difco, Detroit, MI). In the first 4 weeks after injection, the rats were screened twice a week for development of joint inflammation, and paw diameters were measured once a week. During the rest of the 6 month observation period, rats were screened once a week and paws were measured when arthritis developed. Paw diameters were measured with a vernier caliper; hind paws were measured at malleoli, front paws at the wrist. The results were scored as follows: a 1-4 mm increase in the summed diameter for all 4 paws was scored as mild arthritis, a 5-8 mm increase as moderate arthritis, and a 9-12 mm increase as severe arthritis. The standard deviation of summed paw diameters in control rats was 0.65 mm.

Histologic examination

Rats were killed by administration of CO_2. Skinned ankle joints were fixed in 4% formalin for 2 weeks and decalcified in 5% formic acid for 2 more weeks before embedding in paraffin. Seven micrometer sections were stained with hematoxylin and eosin for histologic examination.

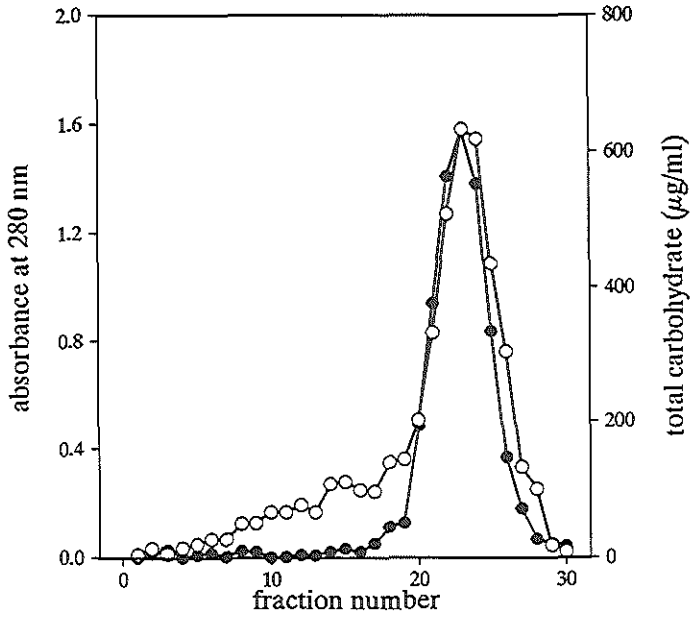

Figure 1. Levels of protein (●) and total carbohydrate (O) in fractions of eluted ethanol precipitate from patient 1 (8 ml fractions eluted for 5 minutes); the void volume was 100 ml.

RESULTS

The presence of peptidoglycan-polysaccharide complexes in ileostomy fluid from 5 patients was investigated by the same methods used for the isolation of peptidoglycan-polysaccharide complexes from feces. Size exclusion chromatography (figure 1) showed that in ethanol precipitate, carbohydrate fractions (fractions 10-19) were eluted before the glycoprotein peak (fractions 20-25). Analysis of the carbohydrate fractions demonstrated the presence of muramic acid (40% of which was connected to interpeptides of peptidoglycan) and rhamnose (table 1); this was therefore considered to be peptidoglycan-polysaccharide complex. A muramic acid content of, for example, 8.7% (as in patient 1, table 1) indicates that about 35% of the sample consists of peptidoglycan, since muramic acid is a compound specific for peptidoglycan and makes up about 25% of the peptidoglycan subunit, as described by Schleifer and Kandler (15).

Peptidoglycan-polysaccharide complexes from the ileostomy fluid of patient 1 had the highest content of muramic acid and rhamnose and were used for further studies, i.e., in several experiments testing for arthritogenicity in rats (table 2). The results show that these peptidoglycan-polysaccharide complexes were able to induce a severe, chronic

Table 1. Chemical composition of peptidoglycan-polysaccharide complexes isolated from the feces of a healthy subject and from ileostomy fluid from 5 patients*.

	Feces from healthy subject	Ileostomy fluid, patient no.				
		1	2	3	4	5
Muramic acid	17	8.7	5.4	5.7	5.8	5.5
Rhamnose	10	22.2	22.1	12.1	14.0	14.8
Total carbohydrates	35	40	42	36	32	39
Protein	13	1.3	2.0	1.5	2.0	2.6

* Values are expressed as percentage of dry weight of the feces or ileostomy fluid preparation.

arthritis, although the incidence was low. Peptidoglycan-polysaccharide complexes from 2 different batches both induced arthritis. The different isolation procedures used resulted in no statistically significant difference in arthritogenicity (by chi-square test). However, with peptidoglycan-polysaccharide complexes from the second batch, we found a significantly lower rate of incidence ($P=0.05$) and a longer latency period, compared with peptidoglycan-polysaccharide complexes from the first batch (table 2).

Some differences in muramic acid and rhamnose content were found between the 2 batches. The muramic acid content in the second batch was 7.5% after chromatography and 7.2% after ultrafiltration (versus 8.7% after each procedure in the first batch); in both preparations, 40% of the muramic acid was coupled to peptide side chains. The rhamnose content was 20.1% and 14.1% after chromatography and ultrafiltration, respectively, in the second batch (versus 22.2% in the first). In our opinion, these slight differences between both batches do not explain the differences in arthritis induction.

Table 2. Arthritogenicity of peptidoglycan-polysaccharide complexes isolated from the ileostomy fluid of patient 1.

Experiment no.	Batch no./ isolation procedure*	No. of rats	No. that developed arthritis	Time to onset (weeks)	pattern**
1	1/chrom.	5	2	3-7	A/A
2	1/chrom.	5	2	3-5	C/B
3	1/chrom.	8	2	4-5	A/A
4	2/ultrafil.	10	1	13	A
5a	2/chrom.	8	0		
5b	2/ultrafil.	8	1	28	A

* Peptidoglycan-polysaccharide complexes from batch 1 or batch 2 were isolated by chromatography (chrom.) or ultrafiltration (ultrafil.).

** A = arthritis of ankle and/or wrist;
B = arthritis of smaller joints of paws;
C = arthritis of ankle/wrist together with smaller joints.

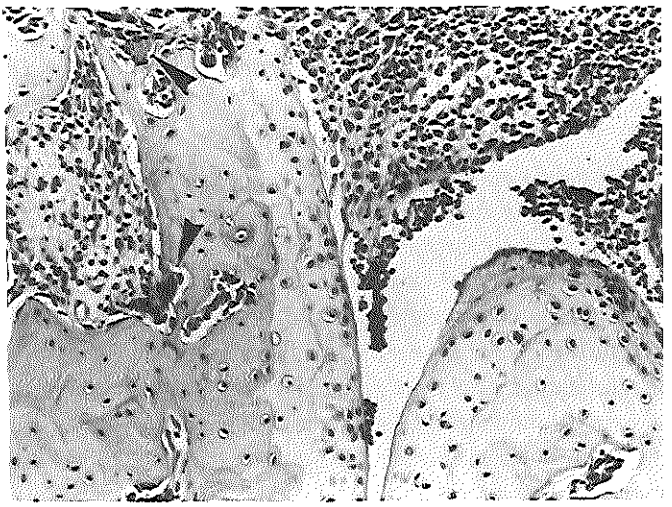

Figure 2. Histologic findings in the paw of a rat injected with peptidoglycan-polysaccharide complexes from precipitate from patient 1 (8 ml fractions eluted for 5 minutes); the void volume was 100 ml. patient 1, 50 days after the first signs of inflammation. A polymorphonuclear infiltrate is visible in the joint space, the synovial tissue is infiltrated by lymphocytes, and erosion of cartilage and bone is seen at the margin, with pannus overlying the cartilage and infiltrating the bone marrow. Arrowheads indicate osteoclasts at sites of erosion (Magnification: 180 x).

Joints of at least 1 rat with arthritis from each experiment were examined histologically. All examined paws showed signs of inflammation. All paws examined showed signs of inflammation. In all arthritic rats, the synovial lining was hyperplastic, with hypertrophic synoviocytes. The synovial tissue was infiltrated by mononuclear cells and, to a lesser extend, by polymorphonuclear cells (PMN). Numerous synoviocytes with large pale nuclei and prominent nucleoli were present. In most rats, fibrosis of the synovial tissue was observed; vascular proliferation did not occur in any of the rats. In the joint space, predominantly PMN were found. The bone itself was heavily damaged: In most cases the cartilage was completely destroyed, and marginal erosions and infiltration of bone by inflammatory tissue were always seen. Bone apposition or new bone formation was seen in most of the animals. A representative histologic section of an inflamed front paw is shown in figure 2. These results further indicated that soluble peptidoglycan-polysaccharide complexes produced by human intestinal flora can induce arthritis in rats.

Peptidoglycan-polysaccharide complexes from the ileostomy fluid of the 4 other patients were subsequently tested for arthritogenicity, using 5 rats for each of the 4 preparations. None of the rats developed arthritis. This is probably because the muramic acid content, or more precisely the content of intact peptidoglycan, was too low.

DISCUSSION

It is difficult to study the etiologic role of intestinal flora in human RA. Fortunately, animal models for arthritis are available. In the adjuvant-induced arthritis, first described by Pearson (16), Wistar or Long-Evans rats developed a chronic arthritis after immunization with Freund's complete adjuvant made of killed *Mycobacterium tuberculosis* ground in mineral oil. In the streptococcal cell wall-induced arthritis, first described by Cromartie *et al.* (17), chronic arthritis was induced in Lewis rats by intraperitoneal injection of cell wall fragments of *Streptococcus pyogenes* in aqueous solution. In both models, translocation of bacterial material to the joint has been described (18,19). In our studies showing the arthritogenic properties of cell wall fragments from *E. aerofaciens* and *B. adolescentis*, major components from human intestinal flora, we used the streptococcal cell wall-induced arthritis model (6,7). Recent studies have shown that cell wall fragments from *E. aerofaciens* also induced chronic arthritis in the adjuvant-induced arthritis model (Kool J *et al.*: manuscript in preparation).

In this study, soluble peptidoglycan-polysaccharide complexes from the anaerobic human bowel flora were tested for arthritogenic properties, using the adjuvant-induced arthritis model. This method was chosen for 2 reasons: First, the amount of material administered is 10-fold lower compared with the intraperitoneal route used for streptococcal cell wall-induced arthritis model, and second, it has been demonstrated by Fox *et al.* (20) that in the streptococcal cell wall-induced arthritis model, purified cell wall components with a similar molecular weight as peptidoglycan-polysaccharide complexes (i.e. 10^5 to 10^6 dalton) failed to induce chronic arthritis.

Soluble peptidoglycan-polysaccharide complexes were isolated from feces from a healthy subject; this subject was chosen because in an earlier study of 10 healthy subjects (9), the peptidoglycan-polysaccharide complexes from his feces contained the highest amount of muramic acid. Although the peptidoglycan-polysaccharide complexes isolated from the feces of this subject contained 17% muramic acid and 10% rhamnose (table 1), it did not induce arthritis in 3 separate experiments, each using 3 groups of 10 rats.

In comparing the properties of the peptidoglycan-polysaccharide complexes with those of cell wall fragments of *E. aerofaciens*, we have found 2 differences. First, the amount of rhamnose is higher in cell wall fragments than in peptidoglycan-polysaccharide complexes. Although in our studies with *Eubacterium* species (7) no significant correlation was found between rhamnose content and arthritogenicity, studies by Lehman *et al.* (21) and Stimpson *et al.* (22) showed that besides the requirement that peptidoglycan be present, a high rhamnose content was essential for induction of arthritis. Second, after careful examination of the muramic acid in peptidoglycan-polysaccharide complexes from healthy subjects, it appeared that in all cases it was present in a hydrolyzed form, i.e., not coupled to the peptide side chains of peptidoglycan. In the adjuvant-induced arthritis model, Kohashi *et al.* (23) have shown that the minimum structure required to induce

arthritis consists of a diamino sugar coupled to L-alanyl-D-isoglutamine via muramic acid. These 2 findings may explain why the peptidoglycan-polysaccharide complexes from the healthy subject were not arthritogenic.

Because the peptidoglycan-polysaccharide complexes of the other healthy subjects contained only a relatively small amount of rhamnose and did not contain muramic acid coupled to peptide side chains, it was considered to be useless to test them for arthritogenicity. We have also investigated peptidoglycan-polysaccharide complexes isolated from the feces of a patient with RA. These complexes also failed to induce arthritis, and low rhamnose content (9%) and muramic acid content (10%), without intact peptide side chains, were again found.

Studies from our laboratory showed that polysaccharide-degrading glycosidases are present in feces (24). The presence of these enzymes, originating from bacterial flora, in combination with a relatively long incubation time in the colon, could be the cause of partial hydrolyzation of peptidoglycan-polysaccharide complexes. We thus hypothesized that in areas more proximal in the intestine, i.e., the terminal ileum, the peptidoglycan-polysaccharide complexes are less processed. Patients with an ileostomy provide a good opportunity to investigate this. It has been shown that in ileostomy fluid, glycosidase activity is much lower compared with that in feces (24,25).

Examination of the bacterial flora in the ileostomy fluid showed that the highest numbers of anaerobic bacteria, among them arthritogenic species, were present in the sample from patient 1, although arthritogenic bacteria were also found in the other 4 ileostomy fluids (25). In this regard, we have shown in an earlier study that in feces of both RA patients and healthy subjects, anaerobic bacteria that can induce arthritis (e.g. *E. aerofaciens*) are present in high numbers (8).

Inhibition assays with an enzyme-linked immunosorbent assay technique using human serum with a high titers against fecal peptidoglycan-polysaccharide complexes (described previously by Hazenberg *et al.*, ref. 10) showed that peptidoglycan-polysaccharide complexes from the ileostomy fluid of patient 1 had a great antigenic similarity to fecal peptidoglycan-polysaccharide complexes from the healthy subject used in the present study (data not shown). We hypothesize that in peptidoglycan-polysaccharide complexes from healthy subjects, the antigenic configuration required for induction of arthritis is present, but the additional chemical composition necessary for persistence of the peptidoglycan-polysaccharide complexes in joints is lacking. Alternatively, it is possible that, although great similarity was found between peptidoglycan-polysaccharide complexes from patient 1 and peptidoglycan-polysaccharide complexes from the healthy subject, there are epitopes that differ and that these epitopes are essential for inducing arthritis. In either case glycosidases may play a prominent role, since they may alter the chemical composition or the epitope involved. No proof of this hypothesis is yet available. However, using the streptococcal cell wall-induced arthritis model, we are currently investigating the influence of rhamnosidase on the arthritis inducing properties of the highly arthritogenic *E.*

aerofaciens cell wall fragments (100% incidence in Lewis rats).

In conclusion, we propose that soluble peptidoglycan-polysaccharide complexes with arthritogenic properties are present in human intestinal contents. These peptidoglycan-polysaccharide complexes are derived from anaerobic flora.

ACKNOWLEDGMENTS

We thank Mr. T.M. van Os for his skillful photographic assistance and Mrs. H.J. Elsenbroek-de Jager for secretarial help. This work was financially supported by the Nationaal Reumafonds (Dutch Rheumatism Foundation).

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CHAPTER 6

ENZYMIC ACTIVITY IN ILEOSTOMY EFFLUENT WITH REFERENCE TO THE CHARACTERISTIC FLORA*

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* *Microb Ecology Health Dis 1991;4:215-22.*

SUMMARY

The ileostomy flora was found to consist of $4x10^9$ bacteria/gm dry effluent (mean) and was composed of about equal numbers aerobes (mainly *Escherichia coli* and *Streptococcus faecalis*), microaerophiles (*S. intermedius*) and a large variety of species of obligate anaerobes. The high blood-group antigenicity of intestinal glycoproteins and the low values or absence of glycosidase activity in ileostomy effluent, indicates that the ileostomy flora does not degrade mucus glycoproteins *in vivo*. However, during prolonged *in vitro* incubation of ileostomy effluents, *N*-acetyl-α-D-galactosaminidase and α-L-fucosidase, blood group A and H degrading enzymes respectively, were induced and caused a substantial loss of intestinal glycoprotein blood group antigens. No inactivation of digestive proteases or leucine aminopeptidase by the ileostomy flora was observed during *in vitro* incubation; feces from healthy subjects, however, did inactivate these enzymes effectively. We conclude that ileostomy effluent harbors bacterial species with the potential to produce glycosidases, but that inactivation of digestive proteases is limited to fecal species which are obviously not present in the ileostomy flora. The significance of these phenomena for the host are discussed.

INTRODUCTION

The anaerobic intestinal flora actively degrades dietary polysaccharides and mucus glycoproteins. Bacterial enzymes involved in the break-down of host-derived and dietary products are glycosidases and proteases (1-4). Furthermore, the bacterial flora of the large intestine is thought to play an important role in the inactivation of digestive proteases (5-7). Changes in composition of the fecal flora may cause alterations in enzymic activity. Previous studies in our laboratory showed that the permanent abnormal flora we found in patients with Crohn's disease (8,9) went together with an increased fecal glycolytic and proteolytic activity (10,11). The combination of the raised level of both glycosidases and of proteases may result in an increased degradation of the intestinal mucus layer, thus breaching the protection of the underlying epithelial cells and causing an increase in intestinal mucosal permeability (12). Furthermore, bacterial glycosidases are involved in the release of toxic compounds from dietary products (13).

Ileostomy effluent offers the investigator an opportunity to study interactions between bacterial flora and host, in the small intestine. The distal ileum in colectomized patients represents an ecological niche that harbors a unique flora (14,15) which is not only different from the fecal flora, both qualitatively and quantitatively, but also differs from the normal ileal flora (16). In the present study, the contribution of the ileostomy flora to the degradation of the intestinal mucus and the modification of dietary residues was investigated by measuring the activity of 24 potential mucus glycoprotein- and plant

polysaccharide-degrading glycosidases on artificial substrates. Furthermore, the degradation of natural substrates, such as human intestinal mucus, was studied by determining the loss of blood-group antigenicity.

Proteolytic activity and leucine aminopeptidase are high in ileostomy effluent; in feces of healthy subjects, host-derived proteases are hardly detectable (11,17). Besides possible resorption by the bowel wall and endogenous inhibition, the colon flora is thought to inactivate these proteases. In this study, the ability of the ileostomy flora to inactivate digestive proteases was compared with that of the fecal flora.

METHODS

Ileostomy samples

Ileostomy effluents were obtained from five adult ileostomists with a conventional ileostomy (aged 38-71 years). They had undergone total colectomy more than 5 years before, for relief of Crohn's disease or ulcerative colitis and were all currently in good health. None of the subjects was on a restricted diet or receiving medication. A 30 minutes to 2 hours collection of 10-25 gram fresh ileostomy effluent was made in plastic bags. Immediately after removal, the bags were transported to the laboratory and processed.

Two samples of ileostomy effluent were obtained from each subject, with at least a 2 month interval, and were examined for flora and enzymic activities and mean values determined.

Microbiological techniques

The ileostomy samples were thoroughly mixed, diluted and seeded aerobically on McConkey (Oxoid Ltd, London, UK), azide (Oxoid), Sabouraud (Oxoid) and blood agar (Oxoid) plates. Anaerobes were cultured in flasks filled with an N_2 (90%)/CO_2 (10%) mixture, as described previously (8,18). The medium, Schaedler Broth (Oxoid), was supplemented with 2% agar (Difco), 0.002% resazurin (BDH, Poole, UK) and 0.025% dithiothreitol (Sigma, St Louis, MO) to maintain a low redox-potential (19). After 2 days of incubation total numbers of aerobes and anaerobes were recorded. All colonies grown in the anaerobic flasks were tested for aerobic growth on blood agar plates. Gram-stain, morphology, carbohydrate fermentations and gas-chromatographically estimated end-products of glucose fermentation were used to identify the isolates to genus or species level. Anaerobic bacteria were classified according to Holdeman *et al.* (20).

Sample preparation and enzyme assays

Within 2 hours of removing the bags the ileostomy effluents were stored at -20°C. Preliminary studies showed no changes in enzyme activities for at least 1 month of storage. Samples were diluted in distilled water, homogenized and sonicated seven times for 2 minutes to disrupt bacterial cells.

Twenty-four hydrolytic enzymes were estimated, 23 by using their *p*-nitrophenyl glycoside substrates (Sigma and Api Lab. Prod. Ltd.) in 0.1M phosphate buffer (pH=6.8), using a modification of the methods described previously (10,11). The samples were diluted up to 10 times in phosphate buffer with 0.1% penicillin to prevent bacterial growth during the experiment. After 2 hours of incubation at 37°C of 100 µl 1mM substrate with 100 µl of ileostomy sample, the reaction was arrested by addition of 800 µl 0.1M NaOH. Samples were centrifuged for 10 minutes at 12,000*g* to remove solid particles and transferred to a flat-well microtiter plate. The absorption was read at 400 nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, Irvine, Scotland) and compared with standard curves obtained from solutions of *p*-nitrophenol in the diluted ileostomy suspensions. One unit of enzyme activity (IU) was defined as the amount which released 1 µmol of *p*-nitrophenol per minute at 37°C.

Neuraminidase activity was measured with *N*-acetylneuramin-lactose (Sigma) as substrate according to the method of Warren (21). One unit of neuraminidase released 1 µmol neuraminic acid per minute at 37°C.

Proteolytic activity was determined as described previously (11). Homogenized and sonicated samples were diluted in 0.1M phosphate buffer (pH=7.6) with penicillin. Samples of 100 µl were incubated with 100 µl 1% (w/v) azocasein (Sigma) in phosphate buffer at 37°C for 1 hour. The reaction was stopped by addition of 200 µl 10% (w/v) trichloroacetic acid; unhydrolyzed azocasein, bacteria and other particles were removed by centrifugation at 12,000*g* during 10 minutes and 200 µl of clear supernatant was transferred to 200 µl of 1M NaOH. Absorption was measured at 450 nm and compared with standard curves obtained from solutions of azocasein. Proteolytic activity was expressed as mg of azocasein hydrolysed in 1 hour.

Leucine aminopeptidase (LAP), an enzyme which does not hydrolyse azocasein, was estimated (11) by incubating 200 µl of the homogenized, sonicated and diluted sample in 0.1M phosphate buffer (pH=6.8) with 200 µl 1.5mM L-leucine *p*-nitroanilide (Sigma) at 37°C. This substrate was prepared by dissolving L-leucine *p*-nitroanilide in methanol (100%) and adding 2 volumes of distilled water. After 30 minutes of incubation the reaction was arrested by addition of 140 µl 30% (v/v) acetic acid. The samples were centrifuged at 12,000*g* for 10 minutes. Absorption of the samples was read at 405 nm and compared with standard curves obtained from solutions of *p*-nitroanilide. One unit of enzyme activity (IU) was defined as 1 µmol of released *p*-nitroanilide per minute at 37°C.

Each sample was tested for non-enzymatic substrate hydrolysis after heating at 80°C

for 10 minutes. Spontaneous substrate hydrolysis was tested by incubation of the substrates with buffer.

Degradation of blood group antigens from ileostomy effluent

Glycoproteins from ileostomy effluents from five subjects and feces from five healthy subjects were obtained by dissolving and homogenizing the samples in distilled water and centrifuging for 30 minutes at 10,000*g*. Clear supernatants were lyophilized and 5% solutions (w/v) in 0.1M phosphate buffer (pH=7.4) made. These solutions contained glycoproteins as well as glycosidases and are hereafter referred to as "glycoprotein solution". The presence of blood group antigens in the glycoprotein solution was estimated in heated samples (10 minutes at 80°C) by determining the titres of A, B and H (lectin) antigens in duplicate hemagglutination inhibition tests as described previously (22). The reciprocal value of the highest dilution of glycoprotein antigen that completely inhibited hemagglutination was defined as the antigen titre. Secretor status of the subjects was estimated in saliva. Blood group antigen degrading properties of the ileostomy glycoprotein solutions were tested by incubating the solutions anaerobically at 37°C for 24 hours; 0.1% penicillin was added to prevent bacterial growth during the experiments. Blood group antigen degrading properties of fecal glycoprotein solutions were estimated by incubation of equal volumes of fecal glycoprotein solutions with ileostomy glycoprotein solutions. Control experiments were performed with glycoprotein solutions that were heated for 10 minutes at 80°C to inactivate bacterial enzymes. During each experiment samples were cultured aerobically and anaerobically to confirm sterility: no growth was observed.

Induction of blood group degrading enzymes was tested for each subject by incubating 50 gram of fresh ileostomy effluent for 24 hours at 37°C under strict anaerobic conditions. Hemagglutination inhibition titres were estimated in heated supernatants of the samples. α-L-fucosidase and *N*-acetyl-α-D-galactosaminidase were assayed after sonication of the samples. Control experiments were performed by incubating autoclaved sterilized ileostomy effluent under identical conditions.

Inactivation of proteases from ileostomy effluent

Proteases and LAP inactivating properties of ileostomy effluents (from five patients) and feces (from five healthy subjects) were compared as follows. One volume of fresh feces was carefully mixed with 10 volumes of freshly prepared ileostomy supernatant (30 minutes, 10.000*g*). The feces-ileostomy supernatant suspension and fresh ileostomy effluent were both incubated under strict anaerobic conditions at 37° for 24 hours. Proteolytic and

LAP activity of the suspensions were measured during incubation. Stability of the intestinal proteases during the experiment was tested by incubating ileostomy supernatant, and ileostomy supernatant with autoclave-sterilized feces (10 minutes, 120°C), both supplemented with 0.1% penicillin to prevent bacterial growth.

Statistics

The coefficient of correlation (r) was calculated based on the least-squares criterion (23).

RESULTS

Ileostomy flora

Table 1 shows the major components of the flora of ileostomy effluents. About equal numbers of anaerobic, microaerophilic and aerobic bacteria were cultured. Facultative organisms such as *Escherichia coli* and *Streptococcus faecalis* were found in almost each individual. The composition of anaerobic flora showed a large person-to-person variation, which was remarkably stable within an individual: the same predominant species were cultured from both samples from each individual.

Enzymic activity

Glycosidase activity was, when detectable, low in all subjects (table 2). Difference in activity between two samples taken from one individual was 5-35%. Ileostomy effluent from subject number 1 contained 15 of the 24 tested glycosidases, but in subjects 4 and 5 only seven enzymes were detected. A linear correlation was found between numbers of demonstrated glycosidases and total bacterial numbers ($r=0.945$; $P=0.01$). Proteolytic and LAP activity were high in each subject.

Degradation of blood group antigens in ileostomy effluent

Intestinal glycoproteins prepared from ileostomy effluents possessed considerable H or H and A blood group antigenicity, in accordance with the subjects' blood group secretor status. No blood group antigens were detected in glycoproteins prepared from feces from healthy subjects.

Table 1. The flora of ileostomy effluent.

	Subject				
	1	2	3	4	5
Total counts*	9.9	9.5	9.9	9.1	9.4
Anaerobes	9.7	9.4	8.8	7.2	9.0
Bifidobacterium adolescentis	9.0	9.2	<4.3	<4.3	<4.3
Clostridium perfringens	9.0	<2.3	<2.3	<2.3	<2.3
Clostridium spp.	<4.3	<4.3	8.2	<4.3	<4.3
Propionibacterium spp.	9.0	<4.3	<4.3	<4.3	8.6
Eubacterium spp.	<4.3	3.4	8.2	<4.3	<4.3
Peptococcus spp.	<4.3	7.2	<4.3	<4.3	<4.3
Lactobacillus fermentum	<4.3	8.8	<4.3	<4.3	<4.3
Peptostreptococcus productus	<4.3	<4.3	8.5	<4.3	<4.3
Veillonella parvula	9.2	8.0	<3.0	<3.0	9.0
Bacteroides vulgatus	<3.0	8.2	<3.03	<3.0	<3.0
Bacteroides spp.	8.5	<3.0	<3.0	7.2	<3.0
Fusobacterium spp.	8.4	<3.0	<3.0	<3.0	7.9
Microaerophiles					
Streptococcus intermedius	9.6	<4.0	9.8	<4.0	9.0
S. agalactiae	<4.0	8.2	<4.0	<4.0	<4.0
Aerobes	8.9	8.0	8.6	9.2	8.6
Escherichia coli	8.3	7.7	7.8	8.2	8.6
Proteus vulgaris	<4.0	<4.0	<4.0	5.0	<4.0
other *Enterobacteriaceae*	8.2	6.8	<4.0	<4.0	<4.0
Enterococcus faecalis	8.5	7.6	8.5	7.9	4.9
S. salivarius	<4.0	7.3	<4.0	9.1	<4.0
Staphylococcus epidermidis	7.7	<4.0	<4.0	<4.0	<4.0
Lactobacillus spp.	<2.3	6.7	<2.3	7.9	<2.3
Candida albicans	<2.0	<2.0	3.8	<2.0	4.9
Dry weight of feces**	120	97	88	75	69

* duplicate mean number expressed as $\log_{10}$/gm dry weight of sample

** mg/gm wet ileostomy effluent.

Induction of blood group degrading glycosidases was found during incubation of fresh ileostomy effluents. Figure 1 shows that after 2 and 5 hours of incubation the antigen titre started to decrease in samples with A and H antigenic properties respectively. In fresh ileostomy effluents no enzyme activities were detected on *p*-nitrophenyl glycoside substrates (table 2), but after incubation small amounts of *N*-acetyl-α-D-galactosaminidase (0.01 IU/gm dry weight of sample) and α-L-fucosidase (0.02 IU), blood group A and H antigen degrading enzymes respectively, were detected. It was concluded that the ileostomy flora does not produce blood group degrading enzymes *in vivo* but that these enzymes are produced *in vitro*.

Table 2. Proteolytic, LAP and glycosidase activity of ileostomy effluents from five subjects. Glycosidases are arranged in two main groups, group I being mainly active against intestinal mucus glycoproteins and animal tissues, and group II mainly against dietary carbohydrates.

	Subject				
	1	2	3	4	5
Proteolytic activity*	336	972	355	215	89
LAP**	38	92	26	15	24
Glycosidases**					
Group I					
NAc-α-D-glucosaminidase	0.03	0	0	0	0
NAc-β-D-glucosaminidase	0.25	0.24	0.17	0.26	0.04
α-D-galactosidase	0.11	0.61	0.41	0	0.07
β-D-galactosidase	0.23	0.77	0.36	0	0.19
Phospho-β-D-galactosidase	0.16	0.41	0.24	0	0.13
α-L-fucosidase	0	0	0	0	0
β-D-fucosidase	0.19	0.19	0.32	0.43	0.04
β-L-fucosidase	0	0	0	0	0
NAc-α-D-galactosaminidase	0	0	0	0	0
NAc-β-D-galactosaminidase	0.07	0	0	0	0
Neuraminidase	0.14	0	0	0	0
β-D-glucuronidase	0.04	0	0.09	0	0
α-D-mannosidase	0	0	0	0	0
β-D-mannosidase	0	0	0	0	0
Group II					
α-D-glucosidase	0.18	0.27	0.31	0.13	0.08
β-D-glucosidase	0.11	0.21	0.06	0.06	0
α-maltosidase (α-amylase)	0.10	0.10	0.25	0.10	0.07
β-maltosidase	0.05	0	0.09	0.06	0
β-D-galacturonhydrolase	0	0	0	0	0
α-D-xylosidase	0	0	0	0	0
β-D-xylosidase	0	0	0.11	0	0
α-L-arabinosidase	0.15	0.14	0.28	0	0
β-D-lactosidase	0.11	0	0.07	0.05	0
α-L-rhamnosidase	0	0	0	0	0

* Duplicate mean expressed as mg hydrolysed azocasein/h/gm dry weight of sample

** Duplicate mean expressed as IU/gm dry weight of sample.

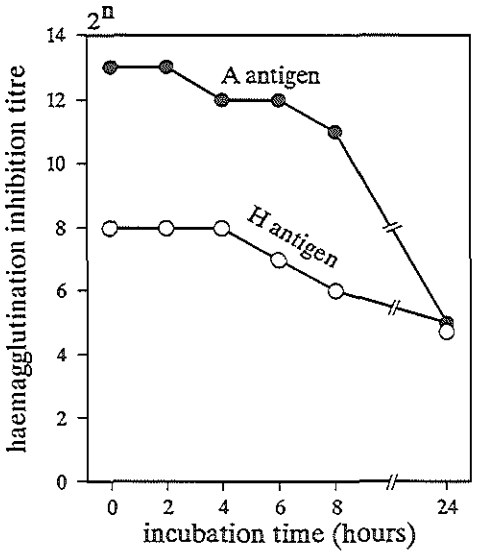

Figure 1. Loss of blood-group antigenicity of intestinal glycoproteins during incubation of ileostomy effluents.

Inactivation of proteases in ileostomy effluent

The addition of a normal fecal flora to ileostomy supernatants caused a decrease of more than 60% in proteolytic activity in 24 hours (figure 2); LAP activity was completely abolished. However, the ileostomy flora had no inhibitory effect at all. Incubation had no influence on the stability of LAP: in bacteria-free ileostomy supernatants and in ileostomy supernatant with sterilized feces the same activity was measured over 24 hours. Proteases appeared to be less stable: a slight decrease in activity was measured during incubation of bacteria-free ileostomy supernatant, which agreed with the decrease in ileostomy effluent. Proteolytic and LAP activity of feces did not influence the results of the experiments; these values were very low, 2.4 ± 0.9 mg hydrolysed azocasein/h/gm wet feces and 0.11 ± 0.05 IU/gm wet feces respectively (median values ± S.D.). From this experiment it was concluded that, unlike the fecal flora of healthy subjects, the ileostomy flora is unable to inhibit digestive proteases.

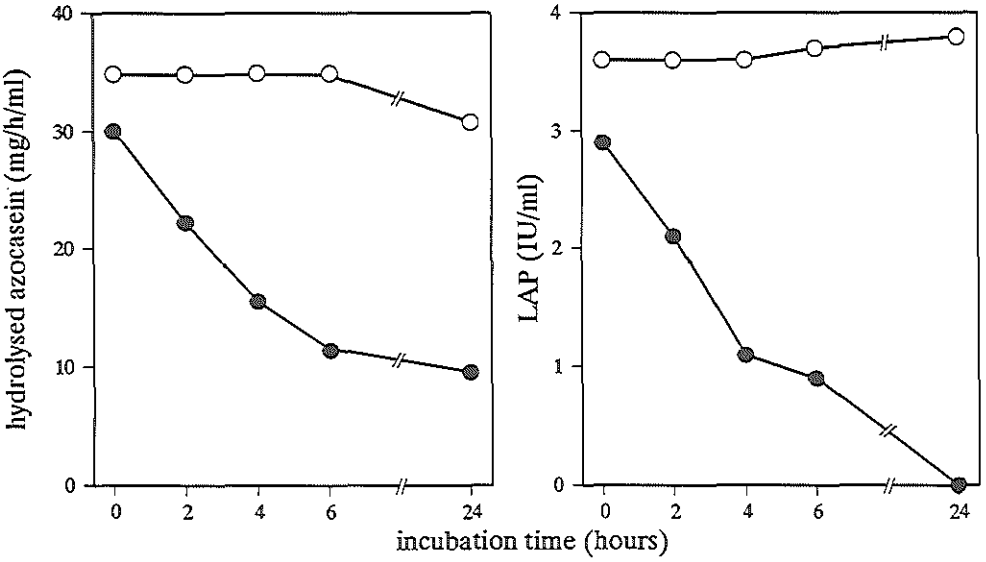

Figure 2. Inactivation of proteolytic enzymes and LAP during incubation of ileostomy effluents (O) and ileostomy supernatants with feces (●).

DISCUSSION

All studies of the composition of the ileostomy flora show similar results for total numbers of aerobes and cultured species, mainly *E. coli* and *S. faecalis*; (14,24,25). However, the ratio of aerobes to anaerobes and the isolated anaerobic species data differ between studies (24,25). Our results are largely in agreement with Kelly *et al.* (15) and Gorbach *et al.* (14) in terms of both the aerobic and anaerobic flora. The large individual variation in anaerobic species that we found is also a characteristic feature of the fecal flora (26). The differences between the ileostomy flora and the colonic or ileal flora are mainly found in the ratio of aerobes to anaerobes, and in total counts. We found total numbers of ileostomy bacteria about 100 times higher than the ileal flora (27), but 10 times lower than the fecal flora (28).

It is likely that the characteristic ileostomy flora is responsible for the low activity or absence of glycolytic enzymes. We previously determined 22 of the 24 tested enzymes in feces of healthy subjects; their activity was about 100 times higher (mean) than in ileostomy effluents measured in the present study (10). Neale *et al.* (29) decontaminated patients with antibiotics, which resulted in strongly reduced glycosidase activity in feces. Feces from germfree rats contain only three glycosidases, ß-D-fucosidase, α-D-glucosidase and α-amylase (30), which are endogenous enzymes of brush-border and

pancreatic origin. Therefore, it is not surprising that these three enzymes belong to the glycosidases that were found in each ileostomy subject. The subject (number 1) with the highest numbers of anaerobes (nearly 10^{10}/gm) had the largest numbers of glycosidases. Probably the small amounts of simple sugars and disaccharides from the diet that escape absorption by the small intestine are normally sufficient for the ileostomy flora to survive. However, an increased flora has to resist food competition by starting to produce glycosidases to split off sugars from larger molecules from dietary and endogenous sources. Non-absorbed dietary carbohydrates are small amounts of starch that are resistant to pancreatic α-amylase (31) and non-starch-polysaccharides (fibre).

The principal endogenous nutrient sources are probably glycoproteins from gastric and small intestinal mucus which contains up to 90% carbohydrate (31,32). Main constituent sugars are *N*-acetyl-glucosamine, galactose, fucose and *N*-acetyl-galactosamine. The high blood group antigenicity of intestinal glycoproteins in ileostomy effluents indicates that hardly any bacterial break down occurs; this is consistent with our findings that none of the ileostomy subjects produced α-L-fucosidase or *N*-acetyl-α-D-galactosaminidase, blood group H and A degrading enzymes respectively. In a previous study we found both enzymes in feces from healthy subjects (10).

Our experiments show that human intestinal glycoproteins are not only readily available to degradation by glycosidases from the fecal flora, but also to the digestive action of ileostomy bacteria after prolonged incubation. We found that it took 2-4 hours of incubation before the flora started to break down blood group antigens from glycoproteins. This was confirmed by the presence of *N*-acetyl-α-D-galactosaminidase and α-L-fucosidase on artificial substrates after 24 hours of incubation. Anaerobic fecal bacteria, such as *Ruminococcus* and *Bifidobacterium* were found to have strong blood group degrading features when cultured on hog gastric mucin (3,33); and *Bacteroides vulgatus* degrades rat intestinal glycoproteins in monognotobionts (22). Not only anaerobes but also aerobic and facultative anaerobic bacteria, such as *Shigella flexneri* were found to split off the B-antigenic determinant from mucus glycoproteins from germfree mouse cecum (34). Thus it seems likely that more than one species of the ileostomy flora is involved in the degradation of intestinal glycoproteins. Blood group degrading enzymes are probably produced when the numbers of bacteria in ileostomy effluent increase during prolonged incubation.

LAP and proteases enter the duodenum largely as secretions from the liver, brush-border and pancreas. A part of the activity is lost in the terminal ileum (11), probably due to absorption and/or action of endogenous inhibitors. In normal feces only a very low enzyme activity can be estimated, which is largely of bacterial origin. It is suggested that the fecal flora is responsible for the inactivation of proteolytic and LAP activity (14,17). This was confirmed by our results: fresh feces were found to markedly decrease these enzyme activities. The ileostomy flora, however, did not change the enzymic activities, indicating that not only is the fast transit of ileostomy effluent responsible for

the high activity, but that no bacteria are present in ileostomy effluent with the ability to inactivate proteolytic enzymes such as trypsin, α-chymotrypsin, elastase and LAP.

The low glycosidase activity of ileostomy effluent, and in particular the presence of high blood group antigen titres of the intestinal glycoproteins, suggest that the mucus layer of the ileum is probably not degraded by bacterial enzymes. As long as the oligosaccharide side chains, which protect the glycoprotein from proteolytic degradation, are intact, even the high level of proteases in the ileum will have little effect. The protective function of the mucus layer in the small intestine of patients with a well-functioning ileostomy is probably not affected by bacterial enzymes. Our experiments showed that increasing numbers of bacteria are accompanied by an increased splitting of the carbohydrate part of the intestinal glycoproteins, which is the initial step in glycoprotein degradation. The protein core, lacking the protection of the carbohydrates is then no longer resistant to proteolysis (34,35). Such a situation might arise due to stasis. Also diet might be important since experiments by Berghouse *et al.* (24) and Fernandez *et al.* (36) showed that high fibre, high fat and high protein diets resulted in higher numbers of bacteria in ileostomy effluents.

In conclusion, we have shown that glycosidase activity can be increased in the ileostomy flora, but that the flora lacks the ability to inactivate digestive proteases.

ACKNOWLEDGEMENTS

This study was supported by grant 28-1542 of the Praeventiefonds, The Hague, The Netherlands.

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CHAPTER 7

DETECTION OF INTESTINAL BACTERIA DERIVED PEPTIDOGLYCAN-POLYSACCHARIDE COMPLEXES IN THE TISSUES OF LEWIS RATS

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SUMMARY

Microorganisms or their products have been put forward by many authors as causative agents in rheumatoid arthritis. The most important source of bacteria is the gastrointestinal tract. In previous studies we therefore directed our research towards the intestinal flora. Cell wall fragments of Gram-positive anaerobic intestinal bacteria (e.g. *Eubacterium aerofaciens*, *Bifidobacterium adolescentis*) and soluble peptidoglycan-polysaccharide complexes from ileostomy fluid were proven to be arthritogenic in the Lewis rat. A prerequisite for arthritis development according to the hypothesis of Bennett, however, is the passage of bacterial debris over the bowel wall. In this study we produced a monoclonal antibody (2E9) directed against peptidoglycan-polysaccharide complexes from human feces. With several immunological techniques the specificity of 2E9 for bacterial products is demonstrated. Using 2E9 in an immunohistological assay we were able to show the presence of bacterial products in macrophages in the red pulp of spleens of conventional Lewis rats. Other lymphoid organs did not stain positively with 2E9. Neonatal and young rats showed no staining of the spleen, but positivity could be induced by injecting peptidoglycan-polysaccharide complexes systemically. We conclude that bacterial fragments, which are potentially arthritogenic, are present in the spleen of conventional rats and must be able to pass the bowel wall.

INTRODUCTION

Many authors have suggested a causative role for microorganisms or their compounds in rheumatoid arthritis. The most important source of bacteria is the gastrointestinal tract. Several authors therefore hypothesized that bacterial products from the intestinal flora pass the bowel wall and give rise to a local or systemic immunerespons-se resulting in a joint inflammation (1-4). In previous studies based on this hypothesis we therefore directed our research towards the intestinal flora. We used a rat model to investigate the arthritis inducing properties of cell wall fragments from Gram-positive anaerobic bacteria, major residents of the human intestinal tract (5-8). Several species, e.g. *Eubacterium aerofaciens* and *Bifidobacterium adolescentis*, occurring in numbers of 10^9/gm feces of healthy subjects as well as rheumatoid arthritis patients (8), proved to be arthritogenic when injected intraperitoneally or subcutaneously in Lewis rats (6,8,9). Also soluble peptidoglycan-polysaccharide complexes isolated from ileostomy fluid were shown to be arthritogenic when injected subcutaneously (10). Thus it appears that antigenic structures which are able to induce arthritis are amply present in the intestinal contents in a soluble form.

In a rat model of arthritis Dalldorf *et al.* (11) demonstrated the presence of streptococcal antigen in synovium, liver, spleen and mediastinal lymph nodes after

intraperitoneal injection of cell wall fragments of *Streptococcus* type A into Sprague-Dawley rats, using a fluorescein-labeled *Streptococcus* type A specific antibody. Anderle *et al.* (12) confirmed these findings for synovium and liver in other rat strains, using the same antibody. With a gas-chromatography, mass-spectrometry method the presence of muramic acid, a compound specific for bacterial cell walls, in liver, spleen and paws of streptococcal injected rats could also be detected (13,14). It is not surprising to find bacterial products in the tissues since rather large amounts were injected (20 mg/animal). In the tissues of control rats no bacterial product was found.

In the adjuvant arthritis model of the rat, induced by subcutaneous injection of small amounts (about 1 mg/animal) *Mycobacterium tuberculosis* or *M. butyricum*, the presence of bacterial products has also been described. Jones and Ward (15) demonstrated the dissemination of ^{14}C-labeled *M. butyricum* from the base of the tail to lymph nodes, lung and feet. Vernon-Roberts *et al.* (16) showed the presence of RITC-labeled *M. tuberculosis* in the lymph nodes, knee joints, lung and spleen after subcutaneous injection in the footpad. Also Glenn *et al.* (17) found bacterial antigen in many tissues, among which feet, liver, lymph nodes and spleen, of ^{3}H-labeled *M. butyricum* injected rats. These studies show that in rat models of arthritis bacterial antigen is present at the site of inflammation. Most probably this is a prerequisite for development of arthritis, although in another model of arthritis the joint inflammation can be induced by a non-bacterial synthetic compound (CP20961). In this model an endogenous immunogen (host?, viral?, bacterial?) was suggested to be responsible (18).

All the above mentioned studies investigated the presence of one specific bacterial antigen in the tissues of injected rats. Sen and Karnovsky, however, in a study on the regulation of sleep by microbial products, investigated the presence of such products in the tissues of naive rats (19). They detected muramic acid in the liver, brain and kidney of Sprague-Dawley rats using a thin layer chromatography method. Since muramic acid is a component specific for peptidoglycan it could be concluded that the latter or its subunits are present in tissues. The distribution of the bacterial products within the organs, however, is not known.

In this study we therefore wanted to investigate the presence and distribution pattern of bacterial products in lymphoid organs and synovium of conventional rats. For this purpose we produced a monoclonal antibody against peptidoglycan-polysaccharide complexes from the intestinal flora of a healthy subject. Enzyme linked immunosorbent assays, immunofluorescence, inhibition assays and affinity chromatography were applied to demonstrate the recognition pattern of the monoclonal antibody. Subsequently several organs of conventional Lewis rats were investigated in an immunohistological assay using this monoclonal antibody.

MATERIAL AND METHODS

Animals

Male BALB/c mice from our breeding colony were used for production of the monoclonal antibody.

Female Lewis rats were purchased from Zentralinstitut für Versuchstierzucht (Hannover, Germany) and used at varying ages. Male and female neonatal Lewis rats, born at our institute from pregnant rats purchased from Zentralinstitut für Versuchstierzucht, as well as fetal Lewis rats, were used at the ages indicated.

Isolation of peptidoglycan-polysaccharide complexes

Peptidoglycan-polysaccharide complexes (PPC) were prepared from feces of a healthy subject, from feces of Lewis rats or from ileostomy fluid from a patient as described before (10,20). In short, feces or ileostomy fluid was suspended in distilled water, cambric gauze filtrated and centrifuged at 5,000*g*. The supernatant was precipitated with 96% ethanol. The precipitate was dissolved in distilled water and dialyzed against water. After centrifugation at 100,000*g* the supernatant was applied to a Fractogel TSK HW75 column (gel bed 700x26 mm; Merck, Darmstadt, Germany) with a high molecular weight fractionation range (550-50,000kD). A high molecular carbohydrate fraction with 5-22% (dry weight) muramic acid, accounting for at least 20-80% peptidoglycan, could be separated from a glycoprotein fraction. The protein contents of the carbohydrate fraction could be lowered from 13% to 1% by incubating the ethanol precipitate 2x24 hours with pronase (5% w/w, 0.1M NH_4-acetate, pH 6.5) before gel filtration.

Production of a monoclonal antibody against peptidoglycan-polysaccharide complexes

Peptidoglycan-polysaccharide complexes isolated from feces of a healthy subject (PPC/HS) were used as immunogen. Male BALB/c mice were injected intraperitoneally with 1 mg of pronase-pretreated PPC/HS in complete Freund adjuvant. After 5 weeks the mice received a booster of 1 mg PPC/HS in incomplete Freund adjuvant. Four weeks later the mice received a second booster. Four days after the second booster two mice were sacrificed and spleen cells were fused with the Sp2/0-Ag14 plasmacytoma cell line (21) in a ratio of 5 : 1, using standard procedures for the production of hybridomas. Fused cells were seeded at a concentration of $5x10^4$/well with $1x10^6$/well, 18Gy irradiated rat thymocytes as feeder cells. Culture supernatant of

a subcloned PPC-specific antibody-producing clone was affinity purified over a rat anti-mouse κ (22) column (CNBr-activated Sepharose 4B column, Pharmacia AB, Uppsala, Sweden) before use.

Enzyme linked immunosorbent assay

Reactivity of the antibodies to PPC/HS, PPC from ileostomy fluid and PPC from feces of Lewis rats was determined in an ELISA as described before (23). In short, 0.3 μg/well of PPC was coated overnight at 50°C in polystyrene microtiter plates. Serial twofold dilutions of culture supernatants were incubated 1 hour at 37°C. As detecting antibody peroxidase-conjugated rabbit anti-mouse immunoglobulin 1:1000 (P260, Dakopatts, Denmark) was used. Substrate, *o*-phenylenediamine/H_2O_2, was incubated for 30-45 minutes. Extinction was measured at 492 nm. For reactivity towards mycobacterial 65kD, 0.2 μg/well 65kD antigen (recombinant *M. bovis* hsp65, kindly provided by Dr. J. van Embden, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, ref. 24,25) was coated 1 hour at 37°C in polyvinylchloride microtiter plates, and the ELISA was performed as described above.

The isotype of the antibodies was determined in a sandwich ELISA as described previously (26). Briefly, 1 μg/well affinity purified rat anti-mouse κ was coated 1 hour at 37°C in polyvinylchloride microtiter plates. Antibodies and isotype-specific alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (1:500, Southern Biotechnology Associates, Inc, Birmingham, UK) were incubated as above. Phosphatase substrate (Sigma 104, St Louis, MO) in 2-amino-2-methyl-1-propanol buffer (pH=9.0) was incubated for 1 hour and extinction was measured at 405 nm.

Immunofluorescence assay

Of 35 anaerobic intestinal bacteria (table 1, described by Hazenberg *et al.*, ref. 23) 10 μg/ml whole cell suspensions were made in phosphate buffered saline (PBS). A droplet of the suspension was air dried onto glass slides and fixed by 3 short exposures to a flame. Undiluted culture supernatant was incubated 1 hour at room temperature. Negative controls consisted of incubation with Sp2/0 supernatant. After washing, a FITC-conjugated goat anti-mouse immunoglobulin (Nordic Laboratories, Tilburg, The Netherlands) was used as second step antibody in a 1:300 dilution. After 1 hour incubation the slides were washed, mounted in Aquamount (BDH, Poole, UK) and screened under a Zeiss epi-fluorescence microscope (Oberkochen, Germany).

Table 1. Anaerobic intestinal bacteria used in immunofluorescence assay.

No	Bacterial species		Immunofluorescence
1	*Eubacterium aerofaciens*	(Dij 5)	-
2	*Eubacterium aerofaciens*	(Dij 51)	-
3	*Eubacterium aerofaciens*	(Mi 62)	-
4	*Eubacterium aerofaciens*	(Mi 64)	-
5	*Eubacterium aerofaciens*	(Fo 6)	-
6	*Eubacterium aerofaciens*	(Fo 71)	-
7	*Eubacterium aerofaciens*	(Fi 91)	+
8	*Eubacterium aerofaciens*	(Ko 60)	-
9	*Eubacterium aerofaciens*	(ATCC 25986)	-
10	*Eubacterium contortum*	(Dij 4)	-
11	*Eubacterium contortum*	(Dij 31)	-
12	*Eubacterium contortum*	(Mi 68)	-
13	*Eubacterium contortum*	(Fi 18)	-
14	*Eubacterium rectale*	(Dij 25)	-
15	*Eubacterium rectale*	(Fi 90)	-
16	*Eubacterium rectale*	(Ko 16)	-
17	*Eubacterium fissicatena*	(Dij 21)	-
18	*Eubacterium cylindroides*	(Dij 18)	-
19	*Eubacterium ventriosum*	(Fo 7)	-
20	*Bifidobacterium infantis*	(Mi 7)	-
21	*Bifidobacterium breve*	(Mi 95)	-
22	*Bifidobacterium catenulatum*	(Ko 4)	-
23	*Bifidobacterium longum*	(Ko 88)	-
24	*Peptostreptococcus productus*	(Fo 20)	+
25	*Lactobacillus minutus*	(Mi 83)	-
26	*Propionibacterium acne*	(Ko 74)	-
27	*Veillonella parvulla*	(Fo 97)	-
28	*Clostridium ramosum*	(Fi 85)	-
29	*Streptococcus morbillorum*	(Ko 73)	-
30	*Sarcina ventriculi*	(Fo 26)	-
31	*Ruminococcus albus*	(Dij 6)	-
32	*Coprococcus comes*	(Me 46)	+
33	*Eubacterium contortum*	(Mc 44)	-
34	*Peptostreptococcus productus*	(C 18)	+
35	*Eubacterium contortum*	(Me 47)	+

Table 2. Monoclonal antibodies used in immuno double staining.

MoAb	specificity	isotype	reference
ED_1	monocytes, macrophages, dendritic cells	IgG1	30
ED_2	resident macrophages	IgG1	30
ED_3	macrophage subpopulation in lymphoid organs	IgG2a	30

Immunohistology

Cryostat sections of organs (5 μm) or ankle joint (10 μm) were fixed with pararosaniline as described before (27). In short, 1 ml 4% (w/v) pararosaniline-hydrochloride (Sigma) in 2M HCl and 1 ml 4% (w/v) $NaNO_2$ were mixed and 194 ml distilled water was added after 1 minute. Sections were fixed for 2 minutes and directly afterwards washed for 10 minutes in PBS with 0.2% bovine serum albumin (PBS/BSA). Endogenous peroxidase activity was inhibited by a 10 minute incubation with 0.5% H_2O_2. The PPC-specific monoclonal antibody was diluted in PBS/BSA to a final concentration of 10 μg/ml. As negative control the isotype-matched monoclonal antibody NS7 (anti-sheep red blood cell) was used (TIB114, ATCC, Rockville, MD). Sections were incubated with 70 μl diluted antibody overnight at 4°C. As second step antibody peroxidase-conjugated rabbit anti-mouse immunoglobulin (P260, Dakopatts) was used in a 1:250 dilution in PBS/BSA with 1% normal rat serum to minimize background staining. After 1 hour incubation the substrate was added for 5 minutes at room temperature. As substrate 3,3'-diaminobenzidine-tetrahydrochloride with $NiSO_4$ as described by Green *et al.* (28) was used. Sections were dehydrated, cleared in xylol and mounted in Entellan (Merck). Positive cells are stained black.

Immuno double staining

For double staining with acid phosphatase, the staining with the monoclonal antibody as described above was done. Before the incubation with the diaminobenzidine substrate, acid phosphatase activity was demonstrated as described by Lojda *et al.* (29). The sections were mounted in Aquamount, without prior dehydration.

For double staining with two monoclonal antibodies pararosaniline-fixed, endogenous peroxidase activity-inhibited cryostat sections were incubated with the PPC-specific monoclonal antibody as described above. As second step a peroxidase-conjugated isotype-specific goat anti-mouse immunoglobulin (Southern Biotechnology Association) was used in a 1:100 dilution. After 1 hour incubation the substrate, 3-amino-9-ethylcarbazole, was added for 15 minutes. The second monoclonal antibody (ED1, ED2 or ED3, table 2) was subsequently incubated overnight at 4°C. An isotype-specific alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Association), diluted 1:100, was incubated 1 hour. The phosphatase substrate was incubated for 30-45 minutes at 37°C and sections were washed and mounted in Aquamount.

Inhibition assays

Both in the ELISA and in the immunohistological staining assay, inhibition assays were performed. Twofold dilutions of the antigen in PBS, starting with 500 μg/ml, were added 1:1 to a 20 μg/ml solution of the purified PPC-specific monoclonal antibody. After 1 hour incubation at 37°C under gentle constant rotation the mixture was centrifuged (10,000*g*) and the supernatant used in an ELISA or immunohistological assay. The antigens used as inhibitors in the ELISA were PPC/HS, whole cell suspensions of *Eubacterium aerofaciens* (Fi91), *E. contortum* (Fi18), *Bifidobacterium catenolatum* (Ko4), and *Peptostreptococcus productus* (Fo20) and cell wall fragments of *E. aerofaciens* (ATCC 25986) treated with lysozyme (egg white, Sigma; 10% w/w, 0.1M sodium acetate, pH=5.0, 24 hours). The percentage of inhibition was calculated as [1-(E_{492} of monoclonal antibody absorbed with antigen divided by E_{492} of sham-absorbed monoclonal antibody)] times 100. In the immunohistological staining, inhibitions were performed with PPC/HS.

Affinity chromatography

Purified PPC-specific monoclonal antibody (8.6 mg) was coupled to Fractogel TSK AF amino 650 (gel bed 3.5 ml;Merck) by standard procedures. The coupled Fractogel was incubated with 3 mg PPC/HS in PBS at room temperature under rotation. The column was rinsed with 100 ml PBS. The PPC was eluted with glycine/HCl (0.1M, pH=2.7) and 4 ml fractions were collected. Muramic acid in the fractions was estimated as described by Hadzija (31) and modified by Hazenberg and de Visser (32).

Culturing of cecal flora

From neonatal and young rats (until 24 days old) the cecum and distal 3 cm of the ileum were taken and disintegrated by whirling in 5 ml Schaedler medium (Oxoid Ltd, London, UK) with glass beads. In rats older than 24 days the cecal contents were suspended in Schaedler medium. Appropriate dilutions were used for culturing. Estimation of total numbers of aerobic, anaerobic and microaerophilic bacteria by culturing on selective and non-selective media has extensively been described by investigators from our laboratory (33).

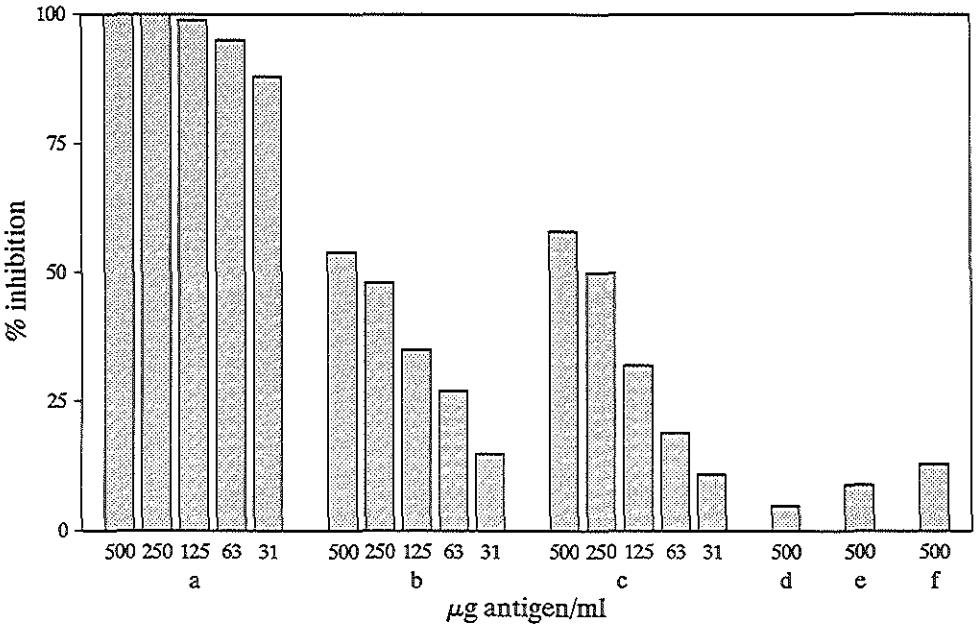

Figure 1. Inhibition by several antigens of the recognition of PPC/HS by 2E9 in an ELISA. Inhibition is expressed as [1-(E_{492} with antigen-absorbed 2E9:E_{492} with sham-absorbed 2E9)] x 100. The antigens used were: (a) peptidoglycan-polysaccharide complexes from feces of a healthy subject, (b) whole cell suspension of *E. aerofaciens* (Fi91), (c) lysozyme treated cell wall fragments of *E. aerofaciens* (ATCC 25986), (d) whole cell suspension of *E. contortum* (Fi18), (e) whole cell suspension of *B. catenulatum* (Ko4), (f) whole cell suspension of *P. productus* (Fo20).

Injection of young rats with PPC

Five week old rats were injected with 1 mg PPC/HS suspended in PBS. Two rats were injected intravenously, two rats intraperitoneally and two non-injected rats served as control rats. After 10 and 17 days one rat of each group was sacrificed and spleen, thymus and paw sections were stained with PPC specific monoclonal antibody as described above.

Arthritis induction with cell wall fragments of *Eubacterium aerofaciens*

Cell wall fragments of *E. aerofaciens* (Fi91) were prepared by a standard procedure as described previously (8). Cell wall fragments were suspended in incomplete Freund

adjuvant in a concentration of 10 mg/ml and 100 µl was injected subcutaneously in the base of the tail of 9 female Lewis rats. Paw sections of arthritic rats were stained with the PPC-specific monoclonal antibody.

RESULTS

Monoclonal antibody against PPC

After fusion growth was found in 9% of the 960 wells, indicating clonality based on the Poisson distribution. Ninety percent of the clones produced antibodies. Twenty-five percent of the producing clones were PPC specific. Of the 21 PPC specific clones 17 clones produced IgM antibodies, 2 clones produced IgG_1 antibodies and 2 clones IgG_3 antibodies. A subclone of one of the IgG_3 producing clones (2E9), obtained by limiting dilution, was selected for further use. Supernatants of 2E9 were purified on a rat anti-mouse κ column before use. 2E9 recognized PPC from a healthy subject (the original immunogen) as well as PPC from ileostomy fluid and PPC from Lewis rats in an ELISA. The 65kD antigen was not recognized in an ELISA. In the immunofluorescence assay 2E9 recognized *E. aerofaciens* (Fi91), *E. contortum* (Me47), *C. comes* (Me46), *P. productus* (Fo20) and *P. productus* (C18). None of the other bacteria was recognized (table 1). The results of the inhibition assays in an ELISA using PPC/HS-coated plates and 2E9 are presented in figure 1. Strong inhibition in a dose-dependent fashion was found with PPC/HS, whole cell suspensions of *E. aerofaciens* (Fi91) and lysozyme treated cell wall fragments of *E. aerofaciens* (ATCC 25986). Whole cell suspensions of *E. contortum* (Fi18), *B. catenolatum* (Ko4), and *P. productus* (Fo20) did not inhibit the recognition of PPC by 2E9 (figure 1). After affinity chromatography of PPC/HS with a 2E9 column, muramic acid could be detected in the eluted fractions immediately after elution with glycine/HCl, showing that 2E9 recognizes a bacterial product. The eluted fraction contained 1/3 of the muramic acid applied to the column. The recovered material differed from PPC/HS in complete absence of rhamnose and protein versus 10% and 13% respectively in the applied PPC/HS. The eluted fraction inhibited the recognition of PPC/HS by 2E9 (ELISA) in a dose dependent way.

Immunohistology of spleens of normal adult rats

Spleens of 15 week old Lewis rats showed staining with 2E9 (figure 2a). Positive cells were localized in the red pulp, with about 8% of all cells in the red pulp being positive. Cells were stained cytoplasmatically.

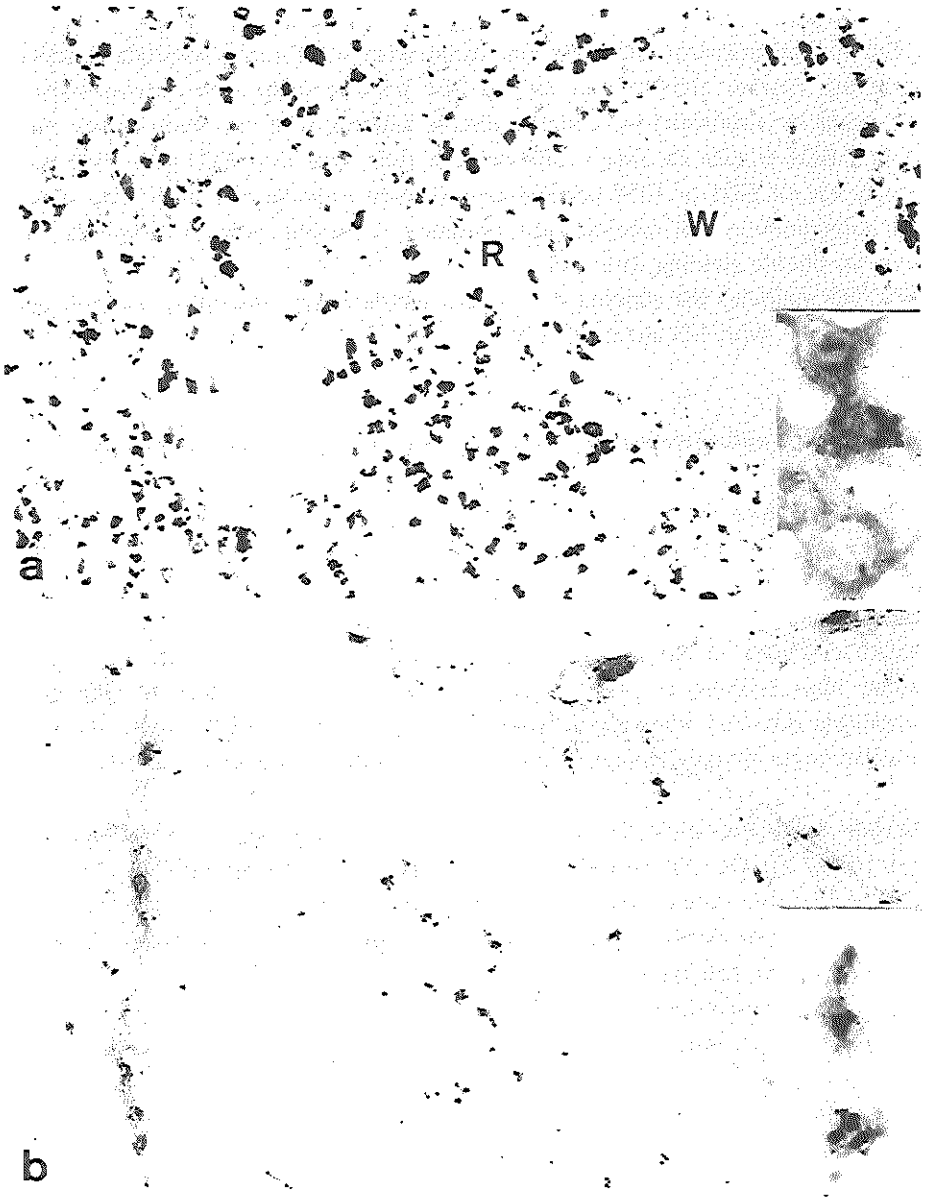

Figure 2. (a) Immunohistologic staining with 2E9 of the spleen of an 15½ week old Lewis rat. R = red pulp, W = white pulp (magnification: 200 x). Inset: 2E9 positive cell (black) double staining with ED2 (gray, magnification: 800 x). (b) Immunohistologic staining with 2E9 of the thymus 1 week after intravenous injection of 1mg PPC/HS (magnification: 200 x). Cells in the parenchyme as well as mast cells in the trabecules stain positively. Inset: 2E9 positive cell in the parenchyme (magnification: 800 x).

The localization of the positive cells suggested that these cells might be macrophages. Staining experiments with 2E9 and demonstration of acid phosphatase activity as macrophage marker showed that indeed 2E9 positive cells were macrophages. This result could be confirmed by immuno double staining with the macrophage markers ED1 and ED2 (figure 2a, inset). 2E9 positive cells were also ED1 and ED2 positive, although not all ED1 or ED2 positive cells showed staining with 2E9. ED3, a marker for a subpopulation of macrophages normally occurring only in lymphoid organs, showed no double staining with 2E9.

PPC/HS inhibited the staining in a dose-dependent way. Inhibition was still present after absorption with 125 μg/ml PPC/HS.

Age dependency and correlation with coccal flora of immunostaining with 2E9

The coccal flora of neonatal and young rats was cultured and the results were compared with the results of immunostaining of the spleen with 2E9 (table 3). In rats less than 10 weeks old, no 2E9 positive macrophages were present. A number of cells in the spleens of rats younger than 5 days, however, showed positive staining of granular structures in the cytoplasm. These cells were shown to be mast cells by toluidine blue staining of serial sections. A very limited number of 2E9 positive macrophages occurred when the rats were 10½ weeks old. At 12½ weeks the number of positive macrophages increased drastically to reach a maximum at 13½ weeks of age.

Already 1 day after birth the first bacteria, being aerobic species, were found in the cecum (table 3). On the second day after birth also microaerophilic species were present. Anaerobic species were not found until the rats were 2 weeks old. Shortly afterwards the total number of anaerobic bacteria became stable although the number of aerobic bacteria still varied.

Rats were decontaminated at the age of 14½ weeks by adding 3 g/l Ampicillin, 2 g/l Neomycin, 2 g/l Polymyxin B and 2 g/l Bacitracin to the drinking water. Ten and 17 days later spleens were stained with 2E9. Although the fecal flora was drastically reduced (table 3, rat number 21 and 22) the number and intensity of 2E9 positive cells remained the same as in not decontaminated rats.

Staining of other tissues

The thymus of Lewis rats showed no staining at any age investigated (from birth to 18 weeks old), except for staining of mast cells in the trabeculas. Also ileal mucosa, Peyer's patches, mesenterial lymph nodes, liver and paw sections of rats were negative.

Table 3. Flora of rats[a] and 2E9 staining of the spleen.

Rat no.	age	total bacteria[b]	total aerobes[b]	total microaerophilic[b]	total anaerobes[b]	spleen staining
1	day 19[c]	ND[d]	ND	ND	ND	–
2	2 hrs	–[e]	–[e]	–[e]	–[e]	–
3	1 day	5.0×10^6	5.0×10^6	–[f]	–[f]	–
4	2 days	3.1×10^8	3.1×10^8	7.1×10^4	–[f]	–
5	3 days	2.3×10^8	9.6×10^6	2.2×10^8	–[g]	–
6	4 days	6.6×10^7	6.3×10^7	2.9×10^6	–[h]	–
7	5 days	8.7×10^8	6.1×10^7	8.1×10^8	–[i]	–
8	10 days	8.7×10^8	6.4×10^8	2.3×10^8	–[i]	–
9	13 days	2.3×10^{10}	5.8×10^9	1.4×10^{10}	2.7×10^9	–
10	17 days	2.5×10^{10}	4.9×10^9	4.1×10^9	1.6×10^{10}	–
11	20 days	1.7×10^{10}	7.9×10^7	2.0×10^9	1.5×10^{10}	–
12	24 days	2.1×10^{10}	3.1×10^6	3.6×10^9	1.7×10^{10}	–
13	32 days	6.3×10^9	3.0×10^5	5.8×10^7	6.3×10^9	–
14	8.5 wk	ND	ND	ND	ND	–
15	9.5 wk	ND	ND	ND	ND	–
16	10.5 wk	ND	ND	ND	ND	±/–
17	11.5 wk	ND	ND	ND	ND	±/–
18	12.5 wk	ND	ND	ND	ND	+
19	13.5 wk	ND	ND	ND	ND	++
20	15.5 wk	6.5×10^9	7.9×10^7	–[i]	6.5×10^9	++
21[j]	16 wk	7.4×10^{7k}	7.4×10^{7k}	–[g]	–[g]	++
22[j]	18 wk	1.4×10^8	1.0×10^8	–[i]	3.5×10^7	++

a. Cecum (rat no. 2-11) or cecal content (rat no. 12, 13, 20-22) were cultured as described in materials and methods.
b. Total numbers expressed per gram cecum (rat no. 1-11) or per gram cecal content (rat no. 12, 13, 20-22).
c. Day 19 of gestation.
d. ND = not done
e. No growth in total cecum.
f. Detection limit 5×10^2/gm.
g. Detection limit 5×10^3/gm.
h. Detection limit 5×10^4/gm.
i. Detection limit 8×10^4/gm.
j. Decontaminated at week 14.5.
k. Only yeasts were cultured.

Staining after injection with PPC

Five week old rats were injected intravenously or intraperitoneally with 1 mg PPC in 2 ml PBS. After 1 and 2 weeks positive staining with 2E9 was found in the spleen and thymus of injected rats, but not in spleen or thymus of age-matched control rats. Positive cells in the spleen were localized in the red pulp and were stained cytoplasmatically, an identical pattern as found in old untreated rats. In the thymus of injected

rats positive cells were localized in the parenchyme (figure 2b). Paw sections were negative in injected as well as non-injected rats.

In intravenously as well as intraperitoneally injected rats immuno double staining of the spleen demonstrated that 2E9 positivity was found in acid phosphatase activity positive, ED1 positive and ED2 positive cells. No double staining with ED3 was found. Immuno double staining of the thymus also resulted in double staining of 2E9 positive cells with ED1 and ED2. This was found for the 2E9 positive cells in the parenchyme of intravenously as well as intraperitoneally injected rats.

Arthritis induction with *Eubacterium aerofaciens* Fi91 and staining of arthritic paws

After subcutaneous injection of cell wall fragments of *E. aerofaciens* (Fi91) 7 of the 9 rats developed arthritis. The onset varied from 21 to 58 days after injection. The arthritis was very severe, both at hind and front paws, and lasted until the end of the experiment (90 days). Three rats with arthritis were sacrificed for immunohistology 4 weeks (1 rat) and 10 weeks (2 rats) after injection. Sections of arthritic paws showed no positive staining with 2E9.

DISCUSSION

In order to find evidence for the presence of bacterial antigens in normal tissues we produced a monoclonal antibody directed to intestinal bacteria-derived peptidoglycan-polysaccharide complexes from feces of a healthy subject. There was a striking distribution over the immunoglobulin subclasses with a predominance of IgM. Also the occurrence of IgG_3 is exceptional. Such a distribution pattern has been described after immunization with group A streptococcal carbohydrate in mice (34). It therefore might be possible that the chosen clone 2E9 recognizes a carbohydrate-containing part of PPC. 2E9 was additionally subcloned and purified before use.

The ELISA and immunofluorescence experiments show that the recognized epitope was present on PPC isolated from feces of a healthy subject as well as on PPC isolated from ileostomy fluid or rat feces and on several intestinal bacteria. The recognition of PPC/HS in an ELISA could be inhibited by PPC/HS as well as whole cell suspension of *E. aerofaciens* (Fi91) and lysozyme treated cell wall fragments of *E. aerofaciens* (ATCC 25986). Affinity chromatography with 2E9 showed that 2E9 reacted with the muramic acid containing fraction of PPC/HS. In this fraction no rhamnose or protein was present. Based on these results we conclude that 2E9 specifically recognizes an epitope present on the glycan backbone of PPC from several anaerobic intestinal bacteria (23).

Using 2E9 we were able to demonstrate the presence of bacterial antigen in the macrophages of the red pulp of the spleen of adult Lewis rats. Specificity of the monoclonal antibody was once more confirmed as after absorption with PPC/HS the staining with 2E9 was inhibited. In rats under 10 weeks of age no staining of macrophages with 2E9 was found. In fetal and neonatal rats until the age of 5 days we found staining of mast cells in the spleen. Also in the trabeculas of thymi mast cells were positive. It is unlikely that this staining is based on recognition of bacterial products, because mast cells are not phagocytic cells. Possibly the staining is based on non-specific charge interactions between 2E9 and the acidic polysaccharides (heparin) in the granules of mast cells. Such interactions with heparin have been described for avidin (35) and for antibodies against *Yersinia* antigens (36). However, no or only weak staining of mast cells was found with the isotype-matched control antibody.

The occurrence of bacterial products in the spleen of naive rats has never been reported before. Sen and Karnovsky (19) demonstrated the presence of muramic acid in brain, liver and kidney of naive rats, but they did not investigate the spleen. Other investigators showed the presence of streptococcal antigen in the spleen of rats after intraperitoneal injection of streptococcal cell wall fragments, but they did not find streptococcal products in the spleen of naive control rats (13) or did not report on the occurrence of such products (12,37). The bacterial products in the macrophages of the spleen may be the "endogenous immunogen" that were supposed to cause arthritis in the CP20961 adjuvant arthritis model (18).

As 2E9 is directed to epitopes on intestinal bacteria we were interested in the 2E9 staining of spleens of fetal and neonatal rats without an intestinal flora or with an establishing intestinal flora. The flora of neonatal rats in our study showed a similar development as described for neonatal rats by Foo *et al.* (38) and Brown *et al.* (39). Aerobes (*E. coli* and *Streptococcus* species) are the first bacteria to settle in the cecum 1 day after birth followed by microaerophilic (*Lactobacilli*, 2 days after birth) and anaerobes (*Bacteroides*, *Peptostreptococcus*, 2 weeks after birth). The ratio of aerobes to anaerobes stabilizes around the third week, with total anaerobes reaching numbers of 10^{10}/gm and numbers of aerobes decreasing to about 10^{7}/gm.

As can be seen from table 3 positive staining of the spleen develops after an intestinal flora has fully developed. In older rats, however, there seems to be no direct relationship between the presence of an intestinal flora and positive staining of the spleen with 2E9. Also decontaminated rats stained positively, although the intestinal flora was drastically diminished or absent. This may be explained by the load of bacterial antigens that is still high in sterilized food as can be seen after Gram staining of a foodpellet (not shown). Thus in all rats that show staining of the spleen with 2E9 bacterial antigen is present. Why younger rats despite a fully developed flora do not stain with 2E9 is not known. The development of the (immune system of the) intestine does not parallel the age at which staining in the spleen becomes positive.

"Closure" of the duodenum for native, non-degraded proteins occurs at about 3 weeks of age (40,41). Peyer's patches obtain a mature structure at 4 weeks after birth (42). And an adult distribution of intraepithelial lymphocytes and lymphocytes in the lamina propria is reached at about 6 weeks (43). Also weaning (circa 3 weeks after birth), sexual development (first ovulation circa week 6, first ejaculation circa week 7) or coprophagia (starting at 16 days and continued throughout life, 44,45) can be ruled out based on the time at which they occur. Thus it remains obscure what (developmental) mechanism is involved.

The requirement for the presence of bacterial products is clear, as is also shown by the positive staining of the spleen that can be induced by injecting a dose of 1 mg of PPC/HS intravenously or intraperitoneally in young rats. Cells in the spleen stain in the same way as in old animals with natural positive staining of the spleen. The bacterial antigen was shown to be present cytoplasmatically in macrophages: acid phosphatase activity positive, ED1 positive and ED2 positive cells in the red pulp. This indicates that the bacterial antigen recognized by 2E9 segregates to these macrophages, not only after injection but also in naive animals. Dalldorf *et al.* (11) described the same distribution pattern of streptococcal antigen in the spleen after intraperitoneal injection of streptococcal cell wall fragments. Vernon-Roberts *et al.* (16) found "mottled" bacterial material in red pulp and marginal zone macrophages of the spleen after subcutaneous injection of RITC-labeled *M. tuberculosis*. The "mottled" appearance shows resemblance with the cytoplasmatic staining in our study.

After systemic injection of PPC/HS staining of thymic macrophages was also found. The thymi of naive rats, however, were always negative (except for mast cells in the trabeculas). Perhaps this is a reflection of the relatively high dose injected or of the route of administration. Passage after systemic injection to the thymus has been described before in mice for antibodies directed to class II or directed to T cells (46), and for antigens (e.g. polyvinylpyrrolidone-coated silica spheres, ref. 47).

Paw sections remained negative after injection of PPC/HS. This might be due to the fact that PPC/HS were injected systemically and were suspended in PBS and not in oil. In their report on distribution of *M. tuberculosis* Vernon-Roberts *et al.* (16) described the presence of bacterial material in the contralateral knee joint when injected subcutaneously in oil, but not after subcutaneous injection in PBS.

Several of the bacterial antigens recognized by 2E9 are arthritogenic in a rat model. In an earlier report we have shown that PPC from ileostomy fluid can induce arthritis after subcutaneous injection in Lewis rats (10). Also lysozyme treated cell wall fragments of *E. aerofaciens* (ATCC 25986) (unpublished results), and cell wall fragments of *E. aerofaciens* (Fi91) as demonstrated in this study induce severe long lasting arthritis after subcutaneous injection. After *E. aerofaciens* (Fi91) injection, the paw sections remained negative when stained with 2E9, a result that is contradictive with findings of other investigators. Dalldorf *et al.* (11) found streptococcal antigen in

arthritic joints, but a higher dose (20 mg versus 1 mg) was injected and the whole joint was used, instead of paw sections, to demonstrate the antigen. Vernon-Roberts *et al.* (16), however, was able to demonstrate antigen in paw sections after injecting an amount of antigen comparable with our amount of *E. aerofaciens* (Fi91). They used antigen that was labeled before injection, which may be a more suitable approach. It is possible that the epitope recognized by 2E9 is altered, but that the fragments that induce arthritis are still present.

How PPC pass the bowel wall and distribute over the body remains obscure. We found no staining of cells in intestinal mucosa, Peyer's patches or mesenterial lymph nodes. It might be possible that bacterial antigens pass the bowel wall extracellularly and distribute over the body as soluble fragments in the lymph and serum. The results from Fox and Fox (48), however, contradict this hypothesis, as they found no muramic acid in the serum of naive rats. But the detection limit (125 μg muramic acid/ml) of their assay may be too high. In a study in mice, Joel *et al.* demonstrated the uptake of carbon particles via M cells and distribution of carbon to Peyer's patches and mesenterial lymph nodes inside macrophages (49). Also other macromolecules may pass the bowel wall via M cells, but their fate after passage of the epithelium is not known. In their study on the distribution of *M. tuberculosis* after subcutaneous injection, Vernon-Roberts *et al.* (16) found that most of the material was transported inside macrophages. We therefore deem it most probable that PPC pass the bowel wall via M cells and distribute over the body inside macrophages.

The transport of bacterial material inside macrophages may be the reason why spleen macrophages stain positive at 12 weeks and not directly after the establishment of an intestinal flora. The mesenterial lymph nodes of mice showed no carbon particles 8 days after start of carbon ingestion, but 2 months later carbon was present within macrophages (49).

In conclusion, we were able to produce a monoclonal antibody that recognizes a muramic acid containing epitope present on PPC of several anaerobic bacteria from the human intestinal flora. With this monoclonal antibody we demonstrated the presence of bacterial antigen in macrophages in the red pulp of the spleen of conventional rats. This indicates that bacterial products are able to pass the bowel wall, but how this occurs remains obscure. Several of the bacterial products recognized by 2E9 have been proven to be arthritogenic, when injected subcutaneously in oil. The next object would be to demonstrate that also in human spleen, and, more interesting, rheumatoid synovium bacterial antigen can be found.

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CHAPTER 8

GENERAL DISCUSSION

8.1 Arthritis and the intestinal flora

Clinical relationships exist between intestinal diseases (ulcerative colitis, Crohn's disease, jejunal bypass syndrome) and arthritic complaints. Also several arthritic diseases (ankylosing spondylitis, reactive arthritis) have been shown to be related to the intestinal flora. In animal studies the arthritogenicity has been demonstrated of cell wall fragments of intestinal aerobic (1) and, numerically more important, anaerobic bacteria in the intraperitoneal model (intraperitoneal injection in rats of cell wall fragments in buffer, ref. 2-5) as well as in the subcutaneous model (subcutaneous injection in rats of cell wall fragments in oil; chapter 4). As in these studies cell wall fragments were used that are artificially prepared from cultured intestinal bacteria we investigated the arthritogenicity of naturally occurring peptidoglycan-polysaccharide complexes (chapter 5). Soluble peptidoglycan-polysaccharide complexes (PPC), originating from the anaerobic intestinal flora (6), can be isolated from faeces and ileostomy effluent. PPC from faeces were not arthritogenic but PPC from ileostomy effluent induced a severe chronic arthritis after subcutaneous injection in rats. PPC from ileostomy effluent differed chemically from faecal PPC: a higher rhamnose content and a higher content of muramic acid coupled to peptide side chains were found in PPC from ileostomy effluent. Rhamnose (1,7) and peptide-coupled muramic acid (8, chapter 5) are thought to be important for arthritogenicity of cell wall fragments. It is likely that the low glycosidase activity and shorter transit time of ileostomy effluent compared with faeces (chapter 6) is responsible for the less degraded, arthritogenic PPC in ileostomy effluent.

Not only the arthritogenicity of intestinal flora derived fragments after injection in rats was investigated, also the influence of the endogenous intestinal flora on arthritis induction was studied (chapter 3). The presence of an autochthonous flora was not necessary for induction of arthritis in the intraperitoneal model in Lewis rats. Pearson *et al.* (9) arrived at the same conclusion for induction of arthritis by subcutaneous injection of *Mycobacterium tuberculosis* in Lobund rats. The role of the intestinal flora in animal models of arthritis seems not to be related with susceptibility to arthritis induction but with induction (or maintenance) of resistance to arthritis. Fischer rats normally resistant to arthritis induction develop chronic arthritis when they are germfree (10,11). Studies in gnotobiotic rats suggest a suppressive effect on arthritis induction for Gram-negative bacteria (lipopolysaccharides?) and an enhancing effect on arthritis induction for Gram-positive bacteria (peptidoglycan?) (12). There remains, however, a controversy on this subject as in animal models lipopolysaccharides are able to induce acute self-limiting arthritis (13) and flare-up reactions of arthritis induced by peptidoglycan-polysaccharides of group A streptococci (14).

Two mechanisms are proposed to explain the involvement of bacteria in rheumatoid arthritis. The first mechanism suggests the induction of an autoimmune reaction by the bacterial antigens, either based on cross-reactivity between bacterial antigens and self-

antigens in the joint, or on the expression of previously hidden self-antigens in the joint. If cross-reactivity exists between the inciting bacterial antigens and self-antigens in the joint, the bacterial antigens may induce a systemic immune response, which is perpetuated by the cross-reactive self-antigens in the joint, leading to chronic arthritis. In the adjuvant-induced arthritis model cross-reactivity has been described between *M. tuberculosis* and the link-protein of proteoglycan (15). There is also a high percentage of homology between mycobacterial and human heat shock protein (16).

Alternatively, the bacterial antigens may induce a local immune reaction which causes destruction of the joint, leading to expression of normally hidden self-epitopes, which also results in chronic joint disease based on autoimmunity. This hypothesis is supported by the existence of a proliferative response of T lymphocytes to syngeneic chondrocytes (17). In either case the persistence of bacterial antigens in the joint is not required for chronicity.

In the second mechanism, bacterial antigens must be present in the joint to induce a local immune reaction leading to arthritis. This mechanism is probably involved in reactive arthritis where bacterial antigens are found in synovial cells (18-20). In rat models of arthritis persistence of bacterial antigens in the joints has been described after injection of bacterial antigens (21-27). Investigation of the presence of bacterial antigens in the joints of rheumatoid arthritis patients with monoclonal antibodies is hampered by the unknown identity of the antigen involved in rheumatoid arthritis. Muramic acid, a component of bacterial cell walls, could not be detected in synovial tissue of rheumatoid arthritis patients (28), but most likely the used method is too insensitive (29). The increased antibody titer to peptidoglycan of streptococci in rheumatoid arthritis patients compared with healthy subjects suggests that bacterial antigen may have passed the bowel wall and distributed over the body (30). However, also in healthy subjects antibodies against bacterial antigens are found (31), indicating that passage of bacterial antigens over the bowel wall may be a naturally occurring phenomenon. Indeed, Sen and Karnovsky (32) demonstrated the presence of muramic acid in liver, brain and kidney of conventional rats. In our study with a monoclonal antibody against PPC the presence of bacterial fragments was demonstrated in spleen macrophages of conventional rats (chapter 7). The most likely route of passage of bacterial fragments over the bowel wall are the M cells overlying Peyer's patches in the ileum (33,34). How the fragments are distributed over the body is unknown. After subcutaneous injection of *M. tuberculosis*, bacterial material is found inside macrophages near the site of injection as well as in spleen macrophages (23). After passing the M cells in the intestine, particles (carbon, latex beads, bacteria) are transported inside macrophages to the mesenterial lymph nodes (35,36). In the thoracic duct of rats dendritic cells could be detected, bearing antigens that were injected intra-intestinally (37). These are indications that bacterial fragments are distributed throughout the body inside macrophages and dendritic cells. Soluble peptidoglycan may also be distributed via the blood. Lichtmann *et al.* (38) described the

presence of peptidoglycan in serum after intraluminal injection in the ileum of rats, suggesting distribution outside cells. Uptake of peptidoglycan in conventional rats is suggested by the presence of anti-peptidoglycan antibodies in serum of conventional rats. Fox and Fox (39) came to an opposite conclusion: they could not demonstrate muramic acid in the serum of conventional rabbits and questioned if uptake of peptidoglycan through the bowel wall really occurs. They did , however, not investigate the presence or absence of muramic acid in the cellular compartment of the blood.

Summarizing, in the human intestine bacterial species are present whose cell wall fragments are arthritogenic in rats. It is likely that potentially arthritogenic peptidoglycan-polysaccharide complexes are present in ileal contents of healthy subjects. Several authors suggest that bacterial antigens may pass the bowel wall, probably via M cells overlying Peyer's patches in the ileum. Indeed we found bacterial fragments in spleen macrophages of conventional rats. Whether PPC are distributed inside macrophages, as soluble molecules in the plasma or otherwise is not clear.

8.2 Arthritis and the immune system

Clinical, histological and *in vitro* experiments indicate an important role for T lymphocytes, especially T helper cells, in the pathogenesis of human rheumatoid arthritis. Also in animal models the CD4 positive T cell is shown to be essential for arthritis induction. In experimental models of arthritis, clones/lines of T cells can transfer the disease (40-42). However, clonality among synovial T lymphocytes in rheumatoid arthritis patients could not be demonstrated unequivocally. Apart from the role of CD4 positive T cells in the induction of arthritis, CD8 positive T cells seem to play a role in the resistance to arthritis induction. Treatment of resistant rat strains with a low dose of cyclophosphamide confers susceptibility to adjuvant-induced arthritis (43). Also treatment of tolerated rats with anti-CD8 monoclonal antibodies results in susceptibility to subsequent arthritis induction (44).

The most likely site of induction of immunity or tolerance to bacterial antigens is the gut. The intestine contains the highest amount of bacteria to which the body is exposed and also constitutes the most likely place for uptake of bacterial antigen in view of its absorptive function. M cells overlying Peyer's patches are thought to play a major role in "sampling" antigens to which mucosal or systemic immune responses should be mounted (33,34). Tolerance to (food) proteins is thought to be achieved via presentation of these proteins by epithelial cells to CD8 positive intra-epithelial cells (45,46). Tolerance induction against particulate/non-protein antigens probably takes place in Peyer's patches (47).

If antigen-specific T lymphocytes are involved in the pathogenesis of arthritis, antigen presenting cells are bound to be involved too. Although the T lymphocyte has attracted

most attention of investigators, the role of antigen presenting cells should not be underestimated. In human rheumatoid arthritis the macrophage-like (type I) synoviocytes are increased in number (48). In synovial tissue increased numbers of class II positive cells, most probably dendritic cells, are found in close proximity of T cells (49). In animal models of arthritis histological investigations of arthritic lesions also show the presence of macrophages (21,50). In *Eubacterium aerofaciens* cell wall fragments induced arthritis (chapter 2) we described an increase in ED1 positive macrophages in synovial tissue that was even more persistent than the increase in T cells. In the streptococcal cell wall-induced arthritis model a rise in peripheral blood monocytes, as well as neutrophils and lymphocytes is found until at least 20 weeks after induction of arthritis. The increase in neutrophils and monocytes correlated with chronic arthritis (51).

Further one must bear in mind that some therapies directed toward T lymphocytes also influence monocytes/macrophages. For example anti-CD4 antibodies induce a 70% decrease in monocyte numbers in the peripheral blood of rheumatoid arthritis patients. Also the increased level of interleukin 1, neopterin and β_2-microglobulin (markers of macrophage activation) in rheumatoid arthritis patients was reduced after anti-CD4 antibody treatment (52). Cyclosporine A has been shown to inhibit macrophage activation by muramyl-dipeptide in guinea pigs (53).

Finally, controversy exists about T cell derived cytokines like interleukin 2 and interferon γ. Most studies fail to show increased amounts of these cytokines in synovial fluid of rheumatoid arthritis patients (54,55). The predominant cytokines found in synovial fluid of rheumatoid arthritis patients are interleukin 1, interleukin 6 and tumor necrosis factor α (54,55). It has been shown that cytokines released by macrophages (interleukin 1 and tumor necrosis factor α) have several effects on cell populations in the joint. These cytokines

- induce proliferation of fibroblast-like (type III) synoviocytes (55,56),
- increase the collagenase production and the interleukin 6 production by fibroblast-like cells (56,57),
- induce expression of ICAM-1 and LFA-3 on endothelial cells, leading to increased adhesion of lymphocytes, especially of CDw29 positive T lymphocytes (54),
- increase adhesion and chemotaxis of neutrophilic granulocytes (55),
- induce osteoclast-mediated bone resorption. However, low doses of interleukin 1 induce osteoblast-mediated bone formation (55,57).

Another cytokine produced by macrophages as well as stimulated fibroblasts is granulocyte-macrophage colony stimulating factor. This cytokine induces increased class II expression, especially of HLA-DQ molecules (58).

Thus many features of human rheumatoid arthritis may be explained by effects of macrophage/fibroblast-derived cytokines.

8.3 Peptidoglycan-polysaccharide complexes and the immune system in arthritis

Peptidoglycan-polysaccharide complexes can exert their effects on the immune system in two ways, both of which may be involved in the pathogenesis of arthritis.

First, PPC has direct effects on the function of several cell types of the immune system. High molecular weight peptidoglycan can induce polyclonal B lymphocyte activation (59,60). Peptidoglycan can also induce activation of the complement system (61,62), of neutrophilic granulocytes (63) and of osteoclasts (64). Smaller fragments of peptidoglycan induce chemotaxis, adherence, proliferation, and activation of macrophages (61). In response to peptidoglycan macrophages produce interleukin 1, hydrolases and proteinases (61,62,65). The minimal structure mediating the effects on macrophages is the *N*-acetyl-muramyl-L-alanyl-D-glutamine moiety (figure 1) present in the peptidoglycan of the majority of the bacteria. Activation of macrophages and B lymphocytes is mediated by binding of peptidoglycan to receptor-like sites on the respective cell types (67,68).

Second, PPC can be processed and presented by antigen presenting cells to T lymphocytes. The antigens presented to T cells are thought to be mainly, if not exclusively, peptides. Peptides of 10-20 amino acids may fit in the peptide-binding groove of major histocompatibility complex molecules; the optimal length of peptides that are able to bind to MHC molecules and to stimulate T cells is 8-12 amino acids (69). It has been shown that different class II molecules have different binding capacities for the same peptide (70). This may explain why rheumatoid arthritis is linked to the expression of the HLA-DR4 gene and the HLA-DR1 gene. The putative antigen-binding site shows only minor conservative changes between Dw4, Dw14 and DR1, but major differences are found with another class II molecule, DR2 (71). The competitive binding of different peptides to the same class II molecule (72) inspired investigators to use non-immunogenic peptides to displace the immunogenic peptide from the class II molecule and thus to prevent the development of a specific T cell response. In this way the development of experimental autoimmune encephalomyelitis can be prevented (73).

It is possible to explain the pathogenesis of arthritis by the two effects of peptidoglycan on the immune system. The peptide side chains of peptidoglycan, which are coupled to muramic acid (figure 1), are 4-5 amino acids long. The side chains are interlinked directly or by additional amino acids, forming bridges between the glycan backbones of peptidoglycan. The peptide side chains are different for the various bacterial species. This may explain why not all bacteria are arthritogenic. The peptide side chains can be released from peptidoglycan enzymatically by *N*-acetyl-muramyl-L-alanine amidase (figure 1). The presence of this enzyme in human serum is described in a limited number of papers (74,75). With the simple and fast assay developed in our department, amidase activity was found in all human sera tested (76). The liberated peptides may have the length that is optimal for binding to major histocompatibility molecules (69). Being an exogenous peptide, presentation will be most likely by class II molecules (77) to CD4

positive T lymphocytes, leading to activation of this T cell subset.

T cells are required to develop arthritis, but it is possible that chronicity is maintained by the presence of activated macrophages. The muramyl peptide moiety of peptidoglycan could be responsible for activation of macrophages. Fibroblasts, neutrophilic granulocytes and osteoclasts can be activated directly by peptidoglycan or indirectly via macrophage-

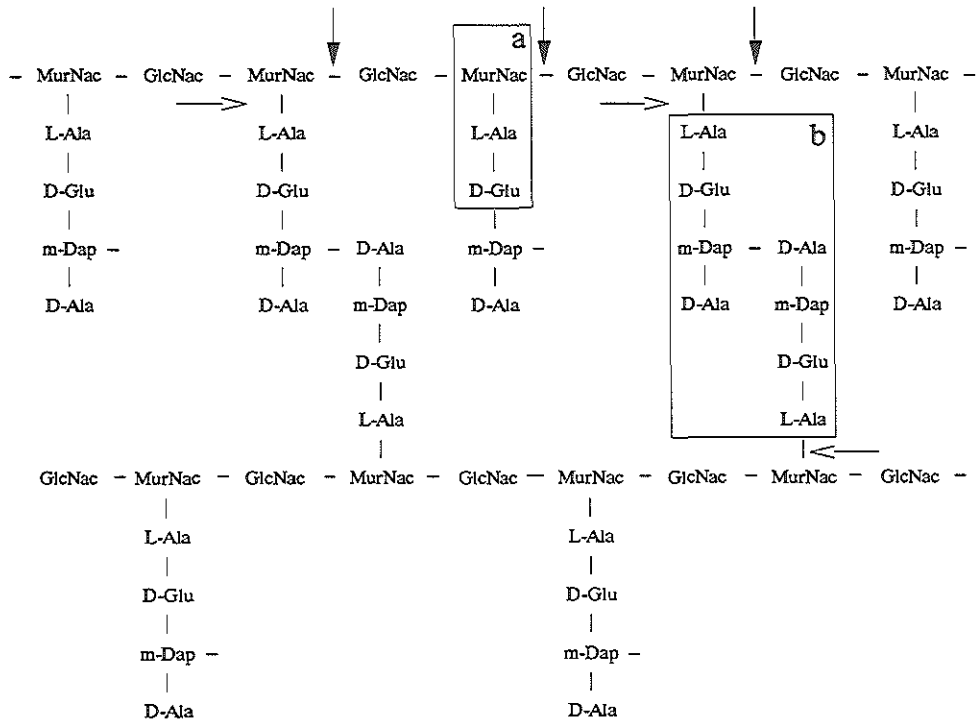

Figure 1. Tentative structure of peptidoglycan in the cell wall of *Eubacterium alactolyticum* (66). The peptidoglycan molecule consists of glycan backbones, interlinked by peptides. The glycan chain is made up of alternating *N*-acetylglucosamine (GlnNac) and *N*-acetylmuramic acid (MurNac) residues. Bound to the MurNac residues are repeating peptide side chains. The composition of the peptide side chains reveals considerable variation between bacterial species. The peptide side chain presented here sequentially contains L-alanine (L-Ala), D-glutamate (D-Glu), meso-diaminopimelic acid (m-Dap), and D-alanine (D-Ala). A peptide bond may be formed between m-Dap of one peptide side chain and D-Ala of another peptide side chain, giving rise to a direct cross-link between two peptidoglycan molecules. Two peptide side chains may also be linked indirectly via additional amino acids.

Hydrolases, present in human serum, cleave specific bonds in peptidoglycan. Lysozyme cleaves the bond between MurNac and GluNac (⟶) and *N*-acetyl-muramyl-L-alanine amidase the bond between MurNac and L-Ala (⟶). (a) muramyl dipeptide moiety; (b) cross-linked peptide side chains, forming peptides with optimal length for binding by major histocompatibility molecules.

derived cytokines. Together these cells are likely to be responsible for the destruction of the joint in chronic arthritis.

Many cytokines are involved in chronic arthritis, therefore cytokine-directed therapy seems not very promising. Therapy directed at the initiating step, the activation of specific T cells, may be more successful. A possibility of specific T cell therapy would be peptide-therapy as described above. However, one must be careful not to use peptides that bind too strong, because this may lead to non-responsiveness to peptides not involved in arthritic disease. A second type of peptide-therapy would be the induction of oral tolerance with the arthritogenic peptides (78) or the induction of bystander suppression with autoantigens from the joint (79,80). The induction of oral tolerance may be the result of the expression of different class II molecules on gut epithelial cells (81) together with a subset of T suppressor lymphocytes in the bowel wall (45). The effectiveness of oral tolerance in arthritis may be high as it has been demonstrated that T lymphocytes from synovial fluid adhere to high endothelial venules of the gut indicating that the synovium and the gut express similar homing receptors for these T lymphocytes (82). A difficulty with both forms of peptide-therapy is that it would only benefit patients with early disease, as in later stages of arthritis especially non-specific mechanisms seem to be involved as well.

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SUMMARY

Rheumatoid arthritis is a chronic, disabling joint disease occurring in about 1% of the population. Women are more often affected than men, and there is a genetic predisposition based on the presence of the HLA-DR4 (Dw4, Dw14, Dw15) gene or the HLA-DR1 gene. Results from investigations in rheumatoid arthritis patients as well as results from experiments in animal models indicate an important role for T helper cells in the disease process.

The causative agent in rheumatoid arthritis is not known. In other arthritic diseases (rheumatic fever, reactive arthritis, ankylosing spondylitis) bacterial antigens, often coming from intestinal bacteria, are involved. Also, intestinal diseases can be complicated by arthritic complaints (ulcerative colitis, Crohn's disease, jejunal bypass syndrome). This led to the hypothesis that in rheumatoid arthritis intestinal flora derived antigens may be involved.

Evidence supporting this hypothesis comes from studies in which cell wall fragments of aerobic and anaerobic Gram-positive intestinal bacteria are shown to induce arthritis after intraperitoneal injection in rats (intraperitoneal model). Especially *Eubacterium aerofaciens* cell wall fragments were able to induce a severe chronic arthritis. Histologically the arthritis resembled human rheumatoid arthritis, although also some differences were observed.

In chapter 2 of this thesis the immunohistological appearance of arthritis induced by *E. aerofaciens* cell wall fragments is described. As in human rheumatoid arthritis, the CD4 positive T cell subset predominated over the CD8 positive subset. B cells and plasma cells were not found. This is in contrast with human rheumatoid arthritis where plasma cells are present, although in lower numbers than T cells. Intriguing was the finding that at the end of the observation period T cells were absent, while ED1 positive macrophages were still present in increased numbers compared with control rats. Clinically the arthritis was still active at this timepoint.

As described in chapter 3, intestinal decontamination of susceptible Lewis rats before induction of arthritis with *E. aerofaciens* cell wall fragments did not change the incidence or the pattern of arthritis. This is in accordance with the unchanged arthritis pattern after subcutaneous injection of *Mycobacterium tuberculosis* in oil (subcutaneous model) in germfree Lobund rats compared with conventional Lobund rats. It appears that the presence of an autochthonous flora is not necessary for the development of arthritis.

In this chapter we also investigated the presence of bacterial species among the intestinal flora of the Lewis rat that can induce chronic arthritis. Although species most likely to induce arthritis were used, none of them gave a chronic arthritis after intraperitoneal injection. Only an acute arthritis was observed. This may explain why we never observed a spontaneous arthritis in Lewis rats.

In the subcutaneous model large as well as small cell wall fragments can be investigated for arthritogenicity in contrast with the intraperitoneal model where only large fragments (i.e. $>5x10^7$ dalton) are able to induce a chronic arthritis. As we were planning to shift our research towards smaller fragments (chapter 5) we first investigated the usefulness of the subcutaneous model using cell wall fragments of *Eubacterium* species. Most *Eubacterium* species gave the same results in the subcutaneous model and the intraperitoneal model. However, *E. rectale* induced chronic arthritis only in the subcutaneous model, and *E. limosum* induced chronic arthritis only in the intraperitoneal model. Despite these differences, it could be concluded that the subcutaneous model is equally suitable for investigating the arthritogenicity of bacterial cell wall fragments as the intraperitoneal model (chapter 4).

In chapter 5 we used the subcutaneous model to study the arthritogenicity of peptidoglycan-polysaccharide complexes (PPC), originating from the intestinal flora. Soluble PPC could be isolated from feces as well as from ileostomy fluid. It appeared that PPC isolated from feces were not able to induce arthritis, while PPC from ileostomy fluid with the highest amount of peptidoglycan induced a severe chronic arthritis in up to 20% of the rats injected. This difference between PPC from feces and ileostomy fluid may be explained by a difference in enzymic activity in the intestinal segments where the PPC originated from. In ileostomy fluid a lower activity of glycosidases compared with feces was found. Also the shorter transit time of ileostomy fluid compared with feces may result in less degraded PPC in ileostomy fluid (chapter 6). It can not be excluded that in ileum of healthy subjects also potentially arthritogenic PPC are present.

Investigators studying the effect of bacterial muramylpeptides in the induction of slow wave sleep suggest that these muramylpeptides are present in mammalian tissues. The presence of muramic acid, a compound specific for the bacterial cell wall, in normal mammalian tissues (liver, brain, kidney) has been demonstrated. In chapter 7 we produced a monoclonal antibody against PPC, shown to react with a muramic acid containing bacterial carbohydrate. We used this antibody to demonstrate the presence of bacterial fragments in the spleen of conventional rats older than 12 weeks of age. The fragments were found in ED1 positive, ED2 positive macrophages of the red pulp. Other tissues (thymus, lymph nodes, intestine, liver, joints) did not stain with the antibody. Thus, bacterial products can be found in the spleen, although it remains obscure how they can pass the bowel wall and reach the spleen.

We found an increased number of macrophages during the whole chronic phase of arthritis induced by cell wall fragments of *E. aerofaciens*. This suggests that macrophages play a major role in the chronic phase of arthritis. Therefore, although the importance of T lymphocytes in the pathogenesis of arthritis is beyond doubt, more attention should be paid to the role of macrophages in the disease process.

SAMENVATTING

Reumatoïde artritis is een invaliderende chronische gewrichtsaandoening, die voorkomt bij ongeveer 1% van de bevolking. Vrouwen lijden vaker aan reumatoïde artritis dan mannen. De ziekte is gerelateerd aan de aanwezigheid van het HLA-DR4 (Dw4, Dw14, Dw15) gen of het HLA-DR1 gen. Uit onderzoek bij reumatoïde artritis patiënten en uit experimenten met proefdieren komt naar voren dat T helper cellen een belangrijke rol spelen in het ziekteproces.

Onbekend is welk agens de oorzaak is van reumatoïde artritis. Bij andere gewrichtsaandoeningen (acuut reuma, reactieve artritis, ziekte van Bechterew) zijn bacteriële antigenen betrokken die vaak uit de darm afkomstig zijn. Tevens zijn er darmaandoeningen (colitis ulcerosa, ziekte van Crohn, jejunum-bypass syndroom) die vaak gepaard gaan met gewrichtsklachten. Dit leidde tot de hypothese dat antigenen afkomstig van darmbacteriën betrokken zijn bij het ontstaan van reumatoïde artritis.

Aanwijzingen voor de juistheid van deze hypothese worden geleverd door onderzoeken waarin de celwand fragmenten van aerobe of anaerobe Gram-positieve darmbacteriën een artritis veroorzaken na injectie in de buikholte van ratten (intraperitoneale model). Vooral celwand fragmenten van *Eubacterium aerofaciens* veroorzaken een ernstige chronische artritis. In histologisch opzicht lijkt deze artritis op reumatoïde artritis bij de mens, hoewel er ook een aantal verschillen zijn.

In hoofdstuk 2 van dit proefschrift wordt het immunohistologische beeld beschreven van artritis veroorzaakt door *E. aerofaciens* celwand fragmenten. CD4 positieve T cellen komen vaker voor dan CD8 positieve T cellen, zoals ook beschreven is voor humane reumatoïde artritis. B cellen of plasmacellen worden niet gevonden. Dit in tegenstelling tot reumatoïde artritis bij de mens waar wel plasmacellen aanwezig zijn, hoewel in lagere aantallen dan T cellen. Intrigerend is dat aan het einde van de observatieperiode T cellen afwezig zijn, terwijl ED1 positieve macrofagen nog steeds in hogere aantallen aanwezig zijn in vergelijking met controle ratten. Op dit tijdstip is de artritis klinisch nog steeds actief.

Zoals in hoofdstuk 3 wordt beschreven heeft decontamineren van Lewis ratten geen effect op de incidentie of het patroon van de artritis veroorzaakt door *E. aerofaciens* celwand fragmenten. Dit is in overeenstemming met het onveranderde artritispatroon na injectie van *Mycobacterium tuberculosis* in olie (subcutane model) in kiemvrije Lobund ratten vergeleken met conventionele Lobund ratten. Uit dit onderzoek blijkt dat de aanwezigheid van een eigen darmflora niet noodzakelijk is voor het induceren van artritis in ratten.

Tevens onderzochten we in dit hoofdstuk de normale darmflora van Lewis ratten op de aanwezigheid van artritis inducerende bacteriën. Hoewel dit onderzoek is uitgevoerd met bacteriestammen die als meest veelbelovend werden beschouwd, veroorzaakte geen van deze stammen een chronische artritis in het intraperitoneale

model. Alleen een acute artritis trad op. Dit kan een verklaring zijn waarom geen spontane artritis wordt waargenomen bij Lewis ratten.

In het subcutane model kunnen zowel grote als kleine celwand fragmenten onderzocht worden op artritis-inducerend vermogen. Dit in tegenstelling tot het intraperitoneale model waarin alleen grote fragmenten ($>5x10^7$ dalton) chronische artritis veroorzaken. Omdat we in een latere fase van het onderzoek kleinere fragmenten wilden gaan bestuderen (hoofdstuk 5), hebben we eerst onderzocht of het subcutane model geschikt is voor het aantonen van het artritis-inducerende vermogen van celwand fragmenten. We hebben daarvoor celwand fragmenten van *Eubacterium* soorten gebruikt. De meeste *Eubacterium* soorten veroorzaken chronische artritis in zowel het subcutane als het intraperitoneale model. Echter *E. rectale* veroorzaakt alleen een chronische artritis in het subcutane model, terwijl *E. limosum* uitsluitend een chronische artritis induceert in het intraperitoneale model. Ondanks deze verschillen kan geconcludeerd worden dat het subcutane model even geschikt is voor het onderzoeken van het artritis-inducerende vermogen van celwand fragmenten als het intraperitoneale model (hoofdstuk 4).

In hoofdstuk 5 hebben we het subcutane model gebruikt om het artritis-inducerende vermogen te onderzoeken van peptidoglycaan-polysaccharide complexen (PPC) afkomstig van darmbacteriën. Oplosbare PPC kunnen worden geïsoleerd uit feces en uit ileostoma vloeistof. PPC uit feces veroorzaken geen artritis, maar PPC uit ileostoma vloeistof met een hoog peptidoglycaan gehalte veroorzaakten chronische artritis in 20% van de geïnjecteerde ratten. Dit verschil tussen PPC uit feces en ileostoma vloeistof zou verklaard kunnen worden door een verschil in enzymactiviteit in het darmsegment waaruit de PPC werden geïsoleerd. In ileostoma vloeistof wordt een lagere glycosidase activiteit gevonden dan in feces. Tevens kan de kortere verblijftijd van ileostoma vloeistof in de darm in vergelijking met feces resulteren in een minder vergaande afbraak van PPC (hoofdstuk 6). Het kan niet worden uitgesloten dat in de dunne darm van gezonde personen ook artritis inducerende PPC aanwezig zijn.

Onderzoekers die het effect bestuderen van bacteriële muramylpeptiden op de "slow wave sleep", suggereren dat deze muramylpeptiden voorkomen in weefsels van zoogdieren. De aanwezigheid van muraminezuur, een bestanddeel dat specifiek is voor bacteriële celwanden, werd aangetoond in weefsel van conventionele ratten (lever, hersenen, nier). In hoofdstuk 7 beschrijven we een monoclonale antistof gericht tegen PPC dat een muraminezuur-bevattend (dus bacterieel) koolhydraat herkent. Met deze monoclonale antistof toonden we de aanwezigheid aan van bacteriële fragmenten in de milt van conventionele ratten ouder dan 12 weken. De fragmenten werden gevonden in ED1 en ED2 positieve macrofagen in de rode pulpa. Andere weefsels (thymus, lymfeklier, darm, lever, gewricht) kleurden niet aan met deze monoclonale antistof tegen PPC. Geconcludeerd kan worden dat bacteriële produkten in de milt

kunnen voorkomen, maar het blijft onduidelijk hoe deze produkten de darmwand kunnen passeren en de milt bereiken.

In ons onderzoek vonden we een verhoogd aantal macrofagen gedurende de gehele chronische fase van de artritis geïnduceerd door *E. aerofaciens* celwand fragmenten. Dit suggereert dat macrofagen een belangrijke rol spelen in de chronische fase van artritis. Hoewel de rol van T lymfocyten in de pathogenese van artritis onbetwist is, zou meer aandacht besteed moeten worden aan de betrokkenheid van macrofagen bij het ziekteproces.

LIST OF ABBREVIATIONS

AcPh	acid phosphatase
ATCC	American Type Culture Collection
B. adolescentis	*Bifidobacterium adolescentis*
B. catenulatum	*Bifidobacterium catenulatum*
B. divaricatum	*Brevibacterium divaricatum*
C. comes	*Coprococcus comes*
C. perfringens	*Clostridium perfringens*
CWF	cell wall fragments
E. aerofaciens	*Eubacterium aerofaciens*
E. coli	*Escherichia coli*
E. contortum	*Eubacterium contortum*
E. lentum	*Eubacterium lentum*
E. rectale	*Eubacterium rectale*
E. limosum	*Eubacterium limosum*
E. tortuosum	*Eubacterium tortuosum*
ELISA	enzyme-linked immunosorbent assay
FITC	fluoresceine isothiocyanate
Gy	Gray
HLA	human leucocyte antigen
ICAM-1	intercellular adhesion molecule 1
IP model	intraperitoneal model
LAP	leucine aminopeptidase
LFA-3	leucocyte function-associated antigen 3
MoAb	monoclonal antibody
M. butyricum	*Mycobacterium butyricum*
M. tuberculosis	*Mycobacterium tuberculosis*
PBS	phosphate buffered saline
PBS/BSA	PBS with 0.2% bovine serum albumin
PMN	polymorphonuclear
PPC	peptidoglycan-polysaccharide complexes
PPC/HS	PPC from feces of a healthy subject
P. productus	*Peptostreptococcus productus*
P. saccharolyticus	*Peptostreptococcus saccharolyticus*
RA	rheumatoid arthritis
RITC	rhodamine isothiocyanate
SC model	subcutaneous model
S. agalactiae	*Streptococcus agalactiae*
S. faecalsi	*Streptococcus faecalis*
S. intermedius	*Streptococcus intermedius*
S. pyogenes	*Streptococcus pyogenes*
S. salivarius	*Streptococcus salivarius*

DANKWOORD

Graag wil ik iedereen bedanken die op enigerlei wijze heeft bijgedragen aan de totstandkoming van dit proefschrift. Een aantal mensen wil ik daarbij met name noemen.

Prof. dr. R. Benner, beste Rob, dank voor de mogelijkheid die je mij hebt geboden om 4½ jaar op jouw Instituut onderzoek te doen. Ook dank dat je mijn promotor hebt willen zijn en voor het in de eindsprint snel, maar vooral ook nauwgezet becommentariëren van mijn manuscript.

Ook de overige leden van de leescommissie, prof. dr. F.T. Bosman, prof. J.H.P. Wilson en dr. W. van Eden, hartelijk dank voor de snelle beoordeling van het manuscript.

Dr. M.P. Hazenberg, beste Maarten, als dagelijks begeleider gaf je mij de ruimte om naar eigen inzicht onderzoek te doen, echter bijtijds bijsturend als dat nodig was. Jouw stroom van ideeën was van grote invloed op het onderzoek dat in dit proefschrift beschreven is. Als, soms zingende, soms pruttelende, motor van ons lab ben je terecht mijn co-promotor.

Ton Severijnen, veel van de practische vaardigheden heb ik bij aanvang van mijn onderzoek van jou geleerd. Meerdere experimenten hebben we vervolgens samen opgezet en uitgevoerd. Menig uur hebben we rattepoten metend in de stallen doorgebracht, waarbij je onderwijl mijn muziekkennis (tevergeefs) trachtte bij te spijkeren. Ik vond het een eer paranymf bij jouw promotie te zijn en ik ben verheugd dat jij mij als paranymf terzijde wil staan.

Marjolein Gerrits-Boeye, sinds maart 1990 ben je intensief betrokken geweest bij mijn onderzoek. Veel van het histologische en immunohistochemische werk is door jou gedaan. Door jou stimulerende invloed c.q. "zoete" dwang (appeltaart weddenschappen!) zijn de resultaten van dat onderzoek vlot op papier gekomen. Behalve als opgeruimde collega ken ik je echter ook als vriendin. Ik zal de spontane "filmmiddagen" in de toekomst missen. Ik ben erg blij dat ook jij mijn paranymf wil zijn.

Ina Klasen, ik zal goede herinneringen bewaren aan onze inspirerende discussies en aan jouw waardevolle commentaren op mijn manuscripten. Ook heb ik genoten van het samen muziek maken op fluit en piano. Ik heb je leren kennen als een uiterst hulpvaardige collega die zichzelf al te vaak wegcijfert.

Johanneke Ruseler-van Embden, ik heb het heel prettig gevonden met je samen te werken aan het ileostoma onderzoek. Ik heb veel gehad aan je opbeurende woorden voorafgaand aan werkbesprekingen en lezingen.

Ook alle overige (ex-)labgenoten hebben stuk voor stuk een bijdrage geleverd aan het proefschrift zoals dat nu voor u ligt: Rik de Visser, Leo van Lieshout, Marie-Jose Melief (HH), Corine van Helden-Meeuwsen. Jullie allen wil ik hartelijk bedanken.

De dagelijkse verzorging van de proefdieren was in de oplettende handen van Lien Braam. Ook de goede samenwerking met Ed Lansbergen (Centraal Proefdier Bedrijf) heb ik zeer op prijs gesteld.

Tar van Os gaf door zijn inzet en perfectionisme dit proefschrift een verzorgd uiterlijk (figuren, foto's, omslag). De lay-out en het drukklaar maken werden in het eindstadium vakkundig verzorgd door Rieke Elsenbroek-de Jager. Beiden namen eerder ook de uiterlijke afwerking van manuscripten voor tijdschriftpublikaties voor hun rekening.

De sfeer op de afdeling en op ons lab in het bijzonder, en de vele "buiten-werkse" activiteiten hebben ervoor gezorgd dat de afgelopen jaren voor mij behalve nuttig en leerzaam ook heel plezierig zijn geweest.

Graag wil ik hier ook een aantal mensen van andere instituten noemen die op een of andere manier belangrijk zijn geweest voor de totstandkoming van dit proefschrift: Wim van den Berg (KUN), Maries van den Broek (CLB, Amsterdam), Jan Rozing (TNO, Leiden), Christien Dijkstra (VU, Amsterdam) en Ilse Vogels (AMC, Amsterdam).

Mijn ouders wil ik bedanken voor de mogelijkheid die ze mij gaven om te studeren, en voor de nodige steun op moeilijke momenten. Zonder hen was ik zo ver niet gekomen. Mijn broer Arjan wil ik bedanken voor zijn belangstelling en voor de goede gesprekken die we hadden.

Familie, schoonfamilie en vrienden/vriendinnen wil ik bedanken voor hun belangstelling voor mijn onderzoek.

Martin, zonder jouw stimulans (die soms meer leek op Ongebreidelde Bikkelharde Pressie) was het schrijven van dit proefschrift nooit zo snel gelukt. Dankzij jouw morele steun bij de laatste loodjes en je relativerende opmerkingen tijdens perioden van (bijna) paniek heb ik de eindstreep zonder al te veel kleerscheuren gehaald.

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