Regulation of bacterial-induced arthritis

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CONTENTS

AA	adjuvant arthritis
APO-SUS	apomorphine susceptible
APO-UNSUS	apomorphine unsusceptible
AVP	arginine vasopressin
CIA	collagen-induced arthritis
CNS	central nervous system
CRF	corticotropin-releasing factor
EAE	experimental allergic encephalomyelitis
FIA	Freund's incomplete adjuvant
GF	germ-free
HLA	human leukocyte antigen
hsp	heat shock protein
HPA-axis	hypothalamus-pituitary-adrenal axis
IL-1/2	interleukin-1/2
LPS	lipopolysaccharide
mAb	mononclonal antibody
MHC	major histocompatibility complex
M.tb.	Mycobacterium tuberculosis
RA	rheumatoid arthritis
ReA	reactive arthritis
RF	rheumatoid factor
SCW	streptococcal cell wall
TNF-α	tumor necrosis factor-α
VLA-4	very late antigen-4

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic, systemic disease affecting about 1-2% of the population. Its aetiology is unknown. The disease is characterized by inflammations mainly in the joints and composed of exacerbations and remissions. The susceptibility to RA is weakly associated with the human leukocyte antigen (HLA) DR4 (1), indicating a genetic predisposition. The disease is more often seen in women than in men (ratio 3:1) (2).

Autoimmunity

Normally the immune system is able to discriminate between "self" and "non-self" (3). As a result, it can respond to an unlimited variety of invading foreign antigens while simultaneously it remains nonresponsive or tolerant to its own self-antigens. In autoimmune diseases however, the immune tolerance against self antigens is broken. Due to this defect autoantibody producing B cells and/or autoreactive T cells are able to attack self-structures.

RA is regarded to be an autoimmune disease even though, besides a so called rheumatoid factor (RF) no crucial autoreactivity has been demonstrated against a particular self antigen. The term RF represents a population of antibodies, which are directed against epitopes on the Fc fragment of autologous IgG (4). They form immune complexes, which can be detected in rheumatoid synovial fluid and tissue. These complexes can fix complement, which points towards involvement in inflammatory reactions (5). RF appears to be frequently present in sera of RA patients, although it can not be considered as the sole criterion for the diagnosis of RA.

Regarding the cellular immunity several studies have already shown that T cells play an important role in RA. One of the first reports on this subject observed that removal of T cells by thoracic duct drainage lead to an improvement of RA (6). Furthermore, reinjection of these removed lymphocytes resulted in a relapse in some patients, as did cessation of the thoracic duct drainage in almost all patients. Later studies showed that also total lymphoid irradiation (7) and cyclosporin A treatment (8) were able to improve RA. Finally, clinical studies are initiated recently to treat RA with the chimeric monoclonal anti-CD4 antibodies cM-T412. The first data show that treatment with anti-CD4 is associated with a sustained decrease in the number of circulating CD4+ T cells. The side effects of anti-CD4 treatment included fever, rigor, chills and hypotension, but no serious infectious complications (9). Regarding clinical improvement the data are not unequivocal. One study reports no clinical improvement, even though histological analysis showed a decrease in the synovial inflammation (10). However, another study reported significant improvement in 12 refractory RA patients (11). It was shown that antibody dosage and treatment regimen are the crucial factors determining clinical outcome. Furthermore, it was observed that the percentage of mAb coated lymphocytes in the synovial fluid correlated with the degree of clinical improvement. It

was suggested that the amount of anti-CD4 monoclonal antibody entering the RA joint may determine clinical efficacy.

Relationship between RA and bacteria

Induction of arthritis is likely to be dependent on the concerted action of three distinct factors: antigen presenting molecules, an arthritis triggering antigen and antigen specific T cells. In this molecular triad the antigen plays an important role for as well the induction as the maintenance of RA. Unfortunately, up to now the antigen is still unknown, although there are several arguments for an involvement of bacteria in the pathogenesis of chronic joint inflammation. For instance arthritis has been associated with bacterial infections of the throat (acute rheumatic fever) (12), the gastrointestinal or urinary tract (Reiter's syndrome, reactive arthritis) (13,14), jejunal bypass surgery for obesity, which often results in a bacterial overgrowth (15), inflammatory bowel diseases like M. Crohn (16) and Borrelia burgdorferi inducing Lyme disease (17). Furthermore several arthritis models exist, which can be induced by the injection of bacteria.

Since RA is a chronic and systemic disease, it indicates that the antigen must be continuously present in either the circulation and/or joint. Combining this with the association between bacteria and arthritis one can think of two likely mechanisms, explaining this autoimmune disease. First of all a specific T cell response against bacterial fragments, which may be present in the circulation or persist in the joint. This persistence in the joint can for instance be explained by trapping of the antigen in immune complexes or by electrostatic interactions of cationic antigens and negatively charged cartilage (18-20). Another likely hypothesis for the induction and chronicity of arthritis is cross-reactivity between epitopes on bacteria or viruses and cartilage.

In reactive arthritis (ReA) the antigens triggering the arthritis are thought to be antigens of the microbes that cause the infections of the gut, such as chlamydia, yersinia or salmonella (21). Antigens of these bacteria have been indeed detected in synovial cells (22-26), and T cell specificity for these antigens has been found in the synovium (27-29).

In RA there are several indications for a direct role of cartilage in the pathogenesis of RA. For instance, destructive forms of rheumatoid arthritis tend to decline at the moment the cartilage is fully destroyed. Moreover, total joint replacement often results in a complete remission of arthritis in that joint. Candidate autoantigens in RA include cartilage proteoglycan aggregates, type II collagen and the heat-shock proteins (hsp). Regarding the human articular cartilage proteoglycans, T cell responses have been found in some patients with RA (30,31), but also in healthy subjects (30-32). Furthermore, these studies showed no simple relationship between T cell responsiveness and disease status. This was also the case for other candidate antigens in RA. For example, T cells responsive to purified type II collagen have been cloned from arthritic joints (33). On the one hand, T cell responses among patients with RA are generally low (34) and T cells from healthy individuals are also able to respond to type II collagen (35). Similarly, studies on hsp as autoantigen have demonstrated that T cell clones from RA inflammatory sites can lyse autologous target cells pulsed with hsp60 (36). In addition, it has been described that RA synovial T cells respond better to members of the hsp60 family than peripheral blood T cells (37). On the other hand, mycobacterium bovis hsp65 is not necessarily an immunodominant T cel antigen in RA (38) and cells from joints of some adult patients with RA do not respond to human hsp60 (39). All these data suggest that

more work is needed to further elucidate, whether reactivity against any of these autoantigens has any relevance in the etiopathogenesis of RA.

ANIMAL MODELS

Since RA is a complicated autoimmune disease, arthritis models have been developed, which closely resemble the human disease. Most of these experimental models are developed in rat or mice and can be divided in unilateral or systemically induced arthritis models. A well known unilateral model is the antigen-induced arthritis, which can be induced by intra-articular injection of antigen into the knee joint of animals previously immunized with the same antigen. In contrast to the unilateral arthritis, the systemically-induced arthritis models result in a polyarthritis. In general, the polyarthritic animal models can be induced in susceptible strains by either structures of the joint itself, like collagen-induced arthritis (CIA)(40) and proteoglycan-induced arthritis (41), by bacteria, like yersinia-induced arthritis (42), adjuvant arthritis (AA)(43) and streptococcal cell wall (SCW)-induced arthritis (44) or by non-immunogenic synthetic oils, like pristane arthritis (45) and CP20961-induced arthritis (46). In addition, there is the spontaneously developing MRL-lpr/lpr mouse model (47). Since a more detailed discussion of all the animal models goes beyond the scope of this thesis, we will now only focus on CIA, AA, SCW-induced arthritis and the CP20961-induced arthritis.

Collagen-induced arthritis

Collagen-induced arthritis (CIA) is an experimentally induced autoimmune model of chronic erosive arthritis. It can be induced in certain strains of rats (40) and mice (48) by intradermal injection of intact ("native") type II collagen, a major component of articular cartilage. Both B and T cells are crucial for the development of arthritis. The importance of antibodies has been demonstrated in experiments in which anti-CII mAb can induce a transient arthritis when transferred to healthy animals (49,50) or nude rats (51). Evidence that T cells play a major role in the development of CIA was obtained from observations, that arthritis does not develop in nude (52) or cyclosporin A-treated rats (53) and that disease can be adoptively transferred with T cell lines or clones (54). Furthermore, the development of disease can be prevented with antibodies directed to CD4 (55) and TCR (56-58). Regarding the pathogenesis, it is suggested that in the active model, binding of anti-CII mAbs to the articular surface may direct the inflammatory process towards this tissue. This may explain the extensive cartilage destruction observed in CIA.

Adjuvant arthritis

Regarding the bacterial-induced arthritis models, most models use either heat-killed bacteria or bacterial fragments. The oldest and most widely used model is adjuvant arthritis (AA). This model is induced by an intradermal administration of heat-killed

Mycobacterium tuberculosis (M.tb.) suspended in Freund's incomplete adjuvant (FIA)(43). The volume, type of oil, and composition of the emulsion are important variables that determine the incidence of arthritis (59,60). Most research on this model has been performed in the susceptible Lewis rat and therefore this will be discussed in more detail. However, AA can also be induced in certain mice (61). In addition, several other studies showed that DA rats injected with FIA alone could develop a mild transient arthritis (62,63), while the Lewis strain remained resistant for this type of induction. In the Lewis rat the polyarthritis develops within 14 days and subsides again after $\pm$ 4 weeks. Interestingly, after this recovery period the Lewis rats are resistant to a renewal injection of M.tb. in oil (64).

In contrast to CIA, AA is primarily a cellular mediated disease. This hypothesis is supported by several studies, which have shown that T lymphocytes play an important role in this arthritis model. For instance, cyclosporin A treatment could prevent the development of disease (65). Furthermore, AA could not be induced in nude rats (66), and was transferable to naive recipients (67). Finally, treatment with the anti-T-lymphocyte antibodies anti-CD4 (68) and anti-TCR (69) were able to prevent arthritis.

Regarding the pathogenesis of this disease, combinations studies on AA and CIA resulted in an extraordinarily severe arthritis (70,71). This synergy provides evidence that AA and CIA are induced by fundamentally different pathogenic mechanisms. In contrast, other studies support the view that at least certain aspects of the pathogenesis of these two models may be the same. For instance, anti-CII antiserum suppressed AA (72) and transfer of spleen cells of donor rats given CII/FIA, which themselves did not induce disease, could suppress the subsequent arthritic response to AA (73).

Interestingly, several data suggest that in AA the pathogenesis is an autoimmune process, which is triggered by structural mimicry between bacterial antigens and cartilage proteoglycans. From studies with the T helper clone (A2b) isolated from AA rats, one was able to pinpoint a reactivity against the 180-186 epitope of the bacterial 65kD heat shock protein (74). Interestingly, this 65kD protein was able to induce an antigen-specific suppression of AA (75,76), and a recent study showed that disease could be inhibited by a peptide analogue of the 180-188 epitope (77).

Reports that AA can be adoptively transferred by T cells were the first indications that crossreactive responses against self-determinants might be involved (67). More recent observations confirm the latter hypothesis, since in the adoptive transfer of AA some of the donor T cells were able to migrate to the synovium (78). Furthermore, the A2b clone was able to respond to cartilage proteoglycan and could induce disease in irradiated syngeneic recipients (79,80). Finally, in vivo AA could be modulated with fractions of proteoglycans (81,82). All these data suggest that crossreactivity between bacterial and cartilage antigens is a likely mechanism for the pathogenesis of this disease. On the other hand, there are several data, which indicate that during the initiation of AA also other mechanisms may play a role. For instance, arthritis can be induced with the non-immunogenic oil CP20961 and this disease is indistinguishable from the classical AA (46). Furthermore, even more surprising are the data that lymphocytes from these CP20961 treated rats recognized the bacterial antigen M.tb. (83). These data suggest that the oil may also induce a second mechanism, which can be described as a nonspecific immunomodulation resulting in expression of normally suppressed autoimmunity.

Inbred rat strains have been found to differ in their susceptibility to AA, indicating that genetic factors control the susceptibility to AA. For example, Lewis rats are highly susceptible to AA, while F344 (Fisher) rats, despite the fact that they share the same

RT1.B and RT1.D MHC class II loci with Lewis, are not susceptible to AA (84). Interestingly, F344 rats kept and bred under germ-free conditions are susceptible to AA (85), whereas colonization of the germ-free rats with gram-negative bacteria again suppressed the disease susceptibility (86). The relevance of these findings are discussed below.

Streptococcal cell wall-induced arthritis

Another important bacterial arthritis model is the streptococcal cell wall (SCW)-induced arthritis. It can be induced by an intraperitoneal injection of a sterile, aqueous suspension of bacterial cells or cell wall fragments from group A streptococci (44). In addition, a similar disease can be induced with cell wall fragments of other bacteria, such as Lactobacillus casei or Eubacterium aerofaciens (87,88). The latter gram-positive bacteria are the major residents of the human intestinal flora. This indicates that large amounts of potentially arthritogenic stimuli are continuously present in the gastrointestinal tract.

Injection of this poorly biodegradable material results in an acute systemic illness together with swelling and redness of the joints. This acute disease occurs after 1 day, wanes after 4-7 days and is dependent on activation of the alternative pathway of complement (89). Acute disease is inducible in all rat strains and in nude or thymectomized rats, indicating independence of functional T cells. The acute phase is followed by a chronic, erosive polyarthritis, which mainly involves peripheral joints. In contrast with the acute phase, the chronic joint inflammation only develops in a limited number of rats strains, namely Lewis and Sprague Dawley rats (90,91).

In the SCW-induced arthritis persistance of the poorly degradable fragments is of utmost importance for chronicity, since administration of mutanolysin (a muralytic enzyme which effectively degrades SCW) after injection of the cell walls, resulted in prevention of this chronic joint disease (92). In addition, when chronic inflammation has subsided, arthritis can be reactivated by systemic challenge with homologous or heterologous cell walls or with bacterial lipopolysaccharide (LPS)(93,94). Due to these latter observations the SCW-induced arthritis significantly differs from AA. In line with the previously described models the chronic phase of the SCW-induced arthritis is also T cell dependent. Arthritis could not be induced in athymic (nude) Lewis rats (95) and cyclosporin A was able to effectively inhibit the chronic phase in euthymic Lewis rats (96). Furthermore, a SCW specific T cell line was able to induce arthritis in naive syngeneic recipients (97) and the development of disease could be prevented by treating the animals with antibodies directed against CD4 (98) or the TCR (99).

The susceptibility to this form of arthritis is still not completely understood. As in AA, also in the SCW-induced arthritis F344 rats are resistant when compared to Lewis rats (100). This suggests that susceptibility is not dependent on the MHC genes alone. Furthermore, several other studies showed that susceptibility could not be explained by persistence of the antigen, since in the body and joints this was comparable in both strains. However, due to the importance of T cells in the chronic phase, this difference may be explained at the level of T cell immunity. Regarding to this it was shown that the susceptible Lewis rats were able to mount a marked SCW-specific T cell response against SCW material after various immunization protocols, while the F344 rats were totally unable to do so (101). This unresponsiveness of the F344 rats was not due to a general

defect of the immune system, since control studies with other antigens showed that the F344 rats were capable of mounting distinct T cell repsonses. In addition, it was shown that depletion of T suppressor cells in vitro resulted in a restoration of the T cell response. This demonstrated that active suppression can play an important role in the F344 strain. Of course the immune system of resistant F344 rats will have a good reason not to respond to the SCW material. Again a likely explanation might be the existence of crossreactivity between bacterial epitopes and cartilage proteoglycans. Along this line, the F344 strain simply recognizes the danger of responding to these potential cross-reactive epitopes and as a result the F344 strain remains resistant to SCW-induced arthritis.

There are several mechanisms supposed to be involved in self-tolerance and thus may be indirectly responsible for the resistance of the F344 strain. First of all there is a mechanism, which operates at the level of T cell development and called clonal deletion. In this process T cells, which express the αß T cell receptor with specificity for peptides derived from self structures will be deleted from the repertoire (102). This deletion or programmed cell death is better known as apoptosis. In addition, mechanisms of peripheral tolerance exist and both clonal anergy (103) and active suppression have been postulated to fulfill this role. Regarding the mechanism of anergy, mature self-reactive T cells recognize their specific antigen in the periphery and instead of being activated, these cells become functionally inactivated. This process of clonal anergy appears to be correlated to the inability to produce IL-2 (104). Finally, self-tolerance can be maintained by active suppression (105). In this process T cells with a specificity for suppression inducing epitopes can inhibit the response of T cells to self-antigens.

Several studies have investigated, whether this tolerance against bacterial fragments is acquired early in life through contact with bacteria of the gut flora. Therefore the susceptibility of the germ-free F344 strain was analysed and it was observed that these rats were susceptible to SCW-induced arthritis and were also able to mount a SCW specific T cell response (106). These data in germ-free animals, together with similar findings in AA suggest that the gut flora plays a important role in the induction and maintenance of immunological tolerance to bacterial antigens. However, whether this tolerance against bacterial antigens can be considered as crosstolerance against cartilage fragments is tempting to suggest but remains to be further investigated.

THE NEUROENDOCRINE SYSTEM

Apart from immunological tolerance, recent studies found increasing evidence of interactions of the central nervous system (CNS), the neuroendocrine system and the immune system. In these systems the hypothalamus-pituitary-adrenal (HPA) axis is an important loop, which is able to connect joint inflammation with the brain.

Inflammatory stimuli such as bacterial fragments are able to dramatically increase the production of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) (107,108). Both non-specific proinflammatory cytokines are predominantly produced by macrophages and possess a wide variety of biological activities, of which the activation of the HPA axis is an important one (109,110). Although the precise site of action is unknown, they either directly or indirectly act at the level of the hypothalamus to release corticotropin-releasing factor (CRF) and arginine vasopressin (AVP)(111,112). These neurohormones

are both secreted into the hypophysial portal system and play major and synergistic roles in the regulation of pituitary ACTH secretion, which in turn stimulates the production of glucocorticoids by the adrenal cortex. Along this line the HPA axis can exert a negative feedback on the immune system, and thus may be an important mechanism in the modulation of joint inflammation. How important this may be, can be illustrated by the findings of Sternberg et al. (113). They were able to show that the Lewis rat, which is susceptible for several other autoimmune diseases, has a defect in its ability to biosynthesize CRH in the hypothalamus (114). As a result the Lewis rat was unable to significantly increase its corticosteroid levels when a stressful situation, like the injection of SCW material, was induced. In addition, they showed that the F344 strain could produce high corticosteroid levels to the same stimulus and that these rats became susceptible for SCW-induced arthritis when they were treated with the glucocorticoid receptor antagonist RU 486. From these data it was postulated that the production of high levels of corticosterone is a mechanism of the F344 strain to protect itself from disease development. However, this hypothesis is not in agreement with the observation that the germ-free (GF) F344 rats are susceptible. In contrast to the low CRH production in the Lewis rats, the same rats appear to have an increased AVP production, which probably serves as a mechanism to compensate for insufficient CRH and glucocorticoid secretion (115).

In contrast with the indirect systemic immunosuppressive effects of CRH, a recent study reported that locally produced CRH can exert proinflammatory effects, since anti-CRH treatment could suppress a locally induced inflammation (116). Similarly, local proinflammatory effects may explain the observation that the CRH protein was expressed at high levels in the inflamed joints of Lewis rats and virtually absent in the F344 rat joints (117). Furthermore, these data illustrate the complex interrelationships of the nervous, endocrine, immune and inflammatory systems.

Interestingly, a similar impaired glucocorticoid response has been described for the Obese strain of chickens (118). In addition, it was shown in experimental allergic encephalomyelitis (EAE), an autoimmune disease model for the central nervous system (CNS), that the resistant PVG rats produce higher levels of corticosterone to ether stress than susceptible Lewis rats (119). During this inflammatory process, which normally lasts 5-7 days, an increase in corticosterone appeared to be essential for the recovery phase of this disease, as has been demonstrated after adrenalectomy in Lewis rats (120). Furthermore, adrenalectomized PVG rats became highly susceptible to EAE and steroid replacement therapy was able prevent the fatal outcome of this disease. These data again indicate that the endogenous production of glucocorticoids plays an important role in the regulation of inflammatory processes, like in experimental autoimmune diseases.

Recently, it has been demonstrated that also patients with RA have a defective hypothalamic response to immune and inflammatory stimuli. This was showed in a decreased ACTH/cortisol and increased prolactin production during stress (121,122). Both cortisol and prolactin are hormones which can exert its effects on the immune system, although prolactin augments immune reactivity instead of suppressing it (123). These data again indicate the relationships between the neuroendocrine and immune system. Future research will further elucidate the relevance of the neuroendocrine system in RA and bacterial arthritis models.

AIM OF THE STUDY

In this thesis we explored mechanisms underlying the regulation of susceptibility to bacterial-induced arthritis in various rat strains. The observation that T lymphocytes play an important role in these models is taken as a starting point of this research.

In chapter 2 we investigated the involvement of the CD4+ cells in the induction and maintenance of the SCW-induced arthritis. Therefore CD4+ cells were depleted before the onset of arthritis or in an established disease. In addition, we performed long-term studies to examine mechanisms of resistance to bacterial-induced arthritis.

In chapter 3 we studied the relevance of the in vitro cross-reactive responses between bacterial epitopes and cartilage in the pathogenesis of bacterial-induced arthritis. Therefore we performed a histological analysis in different arthritis models and compared the level of proteoglycan depletion at different timepoints after arthritis induction. Moreover we examined, whether the release of proteoglycan fragments in the synovial fluid was able to elicit an inflammation in Lewis rats, which were previously immunized with SCW/FIA.

In chapter 4 the pathogenesis of bacterial-induced arthritis was further analysed, by performing adoptive transfer experiments. In this study it was tested, whether donor T lymphocytes are capable of specific migration to the joints and subsequently initiate arthritis. These experiments may give indirect evidence, on whether antigen specific T lymphocytes are able to recognize defined antigens in the joint.

Next, we examined the contributing role of the neuroendocrine system in determining the susceptibility to bacterial-induced arthritis. We focused in chapter 5 on active suppression and/or adrenal activity as determinants regulating the resistance against adjuvant arthritis in the Fisher rat. In chapter 6 both systems were further investigated. Using different Wistar lines and both conventional and germfree rats, we analysed the relative importance of the adrenal activity and bacterial flora in determining the susceptibility to bacterial-induced arthritis.

Finally, in chapter 7 it was investigated, whether adjuvant arthritis could be suppressed by blocking the influx of inflammatory cells with antibodies against adhesion molecules.

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CHAPTER 2

Treatment of rats with monoclonal anti-CD4 induces long-term resistance to streptococcal cell wall-induced arthritis

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Treatment of rats with monoclonal anti-CD4 induces long-term resistance to streptococcal cell wall-induced arthritis

To investigate the role of $CD4^+$ cells in the induction and maintenance of streptococcal cell wall (SCW)-induced arthritis, Lewis rats were treated with a monoclonal antibody against rat CD4 (W3/25). Injection before onset of the arthritis resulted in resistance to SCW arthritis. Treatment with anti-CD4 during ongoing arthritis induced an amelioration of the arthritis, demonstrating that $CD4^+$ cells are involved in both the induction and effector phases of the chronic arthritis.

After return of $CD4^+$ cells to normal levels in the circulation, no arthritis occurred in protected rats, despite the continued presence of SCW in the body. Even reinjection of SCW could not induce arthritis in these rats, suggesting that tolerance to SCW had occurred. In addition, these tolerized rats were refractory to actively induced adjuvant arthritis (AA), but were susceptible to passively transferred AA.

Our data imply, that (a) treatment with anti-CD4 plus SCW induces a long-term resistance to SCW-induced arthritis and adjuvant arthritis, (b) SCW and *M. tuberculosis* may use similar mechanisms of regulation of arthritis and (c) active peripheral suppression is not the mechanism of this nonresponsiveness.

1 Introduction

T lymphocytes are considered to have an important role in the pathogenesis of rheumatoid arthritis (RA) and many animal models which resemble various aspects of this human disease. Models for chronic polyarthritis are used, because it is more convenient to study the involvement of T lymphocytes in initiation and maintenance of arthritis this way. For some of these models, *e.g.* adjuvant arthritis (AA), collagen type II-induced arthritis and streptococcal cell wall (SCW)-induced arthritis, the dependence upon T cells has been shown in studies in nude (athymic) rodents [1, 2] and by transferring the disease to naive recipients with T cell lines [3–6] or clones [7, 8]. Another approach to study the role of T lymphocytes is by *in vivo* depletion using (monoclonal) antibodies directed against cell surface determinants on T cells while attempting to induce arthritis. Experiments have been done using prophylactic protocols in collagen type II-induced arthritis [9], in AA [10, 11] and in murine lupus [12]. Patients with RA are also reported to benefit from treatment with anti-CD4 mAb [13]. In addition, we showed that exacerbations, which may be responsible for chronicity of SCW-induced arthritis are fully dependent on $CD4^+$ T cells [14]. Here we report the use of mAb against rat CD4 (W3/25) in SCW-induced arthritis. This chronic, erosive polyarthritis is induced in susceptible Lewis rats by one i.p. injection of a sterile, aqueous suspension of cell walls from *Streptococcus pyogenes*. Prerequisites for chronic disease are persistence of the poorly biodegradable cell walls [15, 16] and functional T lymphocytes [1, 2, 14, 17–19] that are specific for SCW. To investigate the involvement of $CD4^+$ cells in the disease, we depleted $CD4^+$ cells before onset of arthritis or in established disease. Additionally, we performed long-term studies to elucidate the mechanism of (prolonged) resistance to arthritis.

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Abbreviations: **SCW:** Streptococcal cell wall **AA:** Adjuvant arthritis **RA:** Rheumatoid arthritis **aBSA:** Amidated BSA

2 Materials and methods

2.1 Rats

Female Lewis rats were originally obtained from the Zentral Institut fur Versuchstierzucht (Hannover, FRG) and were bred in our own facilities. Rats weighed 125–150 g at the start of the experiments.

2.2 SCW

Streptococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described [1, 20]. The resulting $10\,000 \times g$ pellet was used throughout the *in vivo* experiments, while the $100\,000 \times g$ pellet was used *in vitro*. These preparations contained 11% muramic acid [21]. Bacteria were kindly provided by Dr. Maarten Hazenberg, Erasmus University, Rotterdam, The Netherlands.

2.3 Induction of SCW arthritis

To induce a chronic polyarthritis, rats were injected i.p. with a sterile, aqueous suspension of SCW in a dose of 15 μg muramic acid/gram rat body weight. Arthritis was scored macroscopically by measuring hindpaw thickness with a caliper, and by histology. The increase of paw thickness during the longest experiment (150 days) due to growth of rats never exceeded 1 mm.

2.4 Induction of AA

Lewis rats were injected intracutaneously at the base of the tail with 1 mg heat killed *Mycobacterium tuberculosis* organism (H37Ra Difco Laboratories Inc., Detroit MI) suspended but not emulsified in oil [22]

2.5 Passive transfer of AA

Lewis rats were injected with *M. tuberculosis* suspended in oil in four footpads and at the base of the tail (125 µg/50 µl/injection site) Ten days later draining LN were removed cells were further purified and activated *in vitro* as described [4] Cells were collected and 5×10^8 cells were injected i.v. into recipient rats

2.6 Treatment of rats with mAb

Rats were injected i.p. with 4 mg mouse mAb at days 0 and 3 or at days 13 16 and 19 after arthritis induction The experimental group received W3/25 (mouse IgG_1 anti rat CD4) whereas the control group received BOM22 (mouse IgG_1 anti human CD74 kindly provided by Dr Wil A Allebes University Transfusion Service Transplantation Serology [23])

2.7 Histology of ankle joints

Ankle joints were removed and processed for histology as described [24] Seven micrometer paraffin sections were made and stained with hematoxilin and eosin

2.8 Immunofluorescence

One million splenic T cells (see above) were incubated with 10 ng/ml W3/25 or with BOM22 (control) in PBS supplemented with 0.1% BSA and 0.01% NaN_3 After 30 min on ice cells were washed twice and incubated with 1/50 diluted FITC-goat anti mouse IgG_1 (Nordic Tilburg The Netherlands) After 30 min on ice cells were washed thrice and fluorescence was scored

2.9 DTH reaction

Rats were immunized with amidated BSA (aBSA [25]) emulsified in IFA (100 µg aBSA in each forepaw) and boosted with 2×100 µg aBSA/IFA in the flanks 1 week thereafter At day 15 10 µg aBSA/10 µl PBS were injected into the pinna of the right ear After 24 and 48 h the DTH reaction was measured

3 Results

3.1 Effect of treatment with anti-CD4 on development of SCW-induced arthritis

Rats were injected i.p. on days 0 and 3 with 4 mg W3/25 (anti CD4) or BOM22 (control) Due to this treatment $< 2\%$ $W3/25^+$ cells were detectable in a suspension of splenic T cells at day 4 while at day 19 the percentage of $W3/25^+$ cells was 59% and 62% in the W3/25 group and the BOM22 group respectively (immunofluorescence data not shown) An arthritogenic dose of SCW was given on day 0

Table 1. Histological score of SCW induced arthritis[a]

Treatment of rats			*n*	No of ankles with score of			
SCW at day	mAb at day			0	1	2	3
0	W3/25	0–3	10	0	9	1	0
0	BOM22	0–3	10	0	2	6	2
0	W3/25,	13–16–19	10	3	3	4	0
0	BOM22	13–16–19	10	0	5	5	0
0–83	W3/25,	0–3	4	4	0	0	0
0–83	BOM22,	0–3	4	0	0	1	3

a) Histological score at day 19 after the last SCW injection (*i.e.* day 19 or day 102) Infiltrate and exudate was scored using a scale from 0–3 by two independent observers (MFvdB MCJvB) on coded hematoxylin and eosin stained sections 0 no infiltrate/exudate 3 – large mass of infiltrate/exudate

Treatment with W3/25 in a phrophylactic protocol resulted in virtual absence of SCW induced arthritis (Fig 1) while the control group followed the normal course of arthritis This difference was also confirmed by histology (Table 1) To investigate the effect of anti CD4 in the established disease we injected mAb on days 13 16 and 19 W3/25 induced a decrease of the paw swelling when compared to the control group ($p = 0.008$ on day 23 and on day 30 Wilcoxon rank test) However histological analysis showed

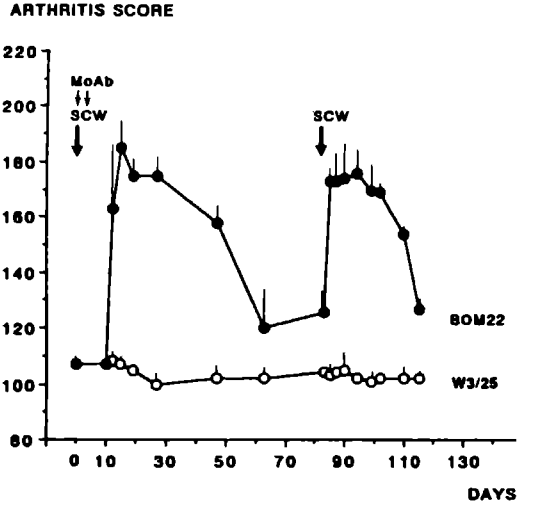

Figure 1 Rats were injected with 4 mg mAb (W3/25 anti CD4 BOM22 subclass control) at days 0 and 3 An arthritogenic dose of SCW was given at days 0 and 83 Arthritis score is expressed as the sum of thickness of both hindpaws (×0.1 mm) Each group consisted of 12 rats

little difference between rats treated from day 13 on with W3/25 or with BOM22 (Table 1)

3.2 Long-term effect of treatment with anti-CD4 on SCW-induced arthritis

Rats were injected on days 0 and 3 with 4 mg W3/25 or with BOM22 (control) SCW arthritis was induced on day 0 in both groups Even when the level of W3/25$^+$ lymphocytes had returned to normal (day 19 and later data not shown), no arthritis occurred in this group, whereas the stimulus (SCW) was presumably still present in the animals [16, 26] To determine whether the newly formed CD4$^+$ cells were refractory to SCW or whether the amount of SCW left in the rats was too low we reinjected rats from both groups with an arthritogenic dose of cell walls (Fig 1) or with one third thereof (= subarthritogenic dose) on day 83 The W3/25 treated group remained resistant to SCW induced arthritis, whereas the control group developed a polyarthritis upon reinjection with SCW with a "normal" severity but with an accelerated onset (1 day after injection *vs* the normal onset on day 12) Paw swellings in rats that received one third of the arthritogenic dose of cell walls were similar to those shown in Fig 1 (data not shown) Thus rats that had developed an SCW-induced arthritis and subsequently recovered displayed an enhanced susceptibility to a further SCW induced arthritis both with respect to SCW dose and to day of onset (BOM22-treated group) In contrast, rats that are made resistant to SCW-induced arthritis by depletion of CD4$^+$ cells plus SCW remained resistant to a further challenge, even after full recovery of CD4$^+$ cells (W3/25-treated group)

Histological analysis confirmed the macroscopically obtained results (Table 1) Injection with rats with anti-CD4 alone on days 0 and 3 followed by an injection with SCW on day 82 does not lead to resistance to SCW-induced arthritis (mean paw thickness on day 20 after SCW W3/25 – 169 ± 12, BOM22 – 173 ± 15)

3.3 AA in rats with a long-term resistance to SCW-induced arthritis

The above data suggest that injection of anti-CD4 together with an arthritogenic stimulus (SCW) results in a long-lasting resistance to arthritis induced by this stimulus To investigate whether these rats were also resistant to arthritis induced by another stimulus we induced AA, or we passively transferred AA at day 62 (Fig 2) The group which received a control mAb and SCW displayed an accelerated onset of arthritis upon *M tuberculosis* administration similar to that seen after reinjection of SCW in this group thus providing evidence for crosspriming W3/25 and SCW-treated rats were refractory to AA This data strongly suggests that despite the fact that AA and SCW induced arthritis were induced by different stimuli the (auto)antigen which is recognized *in vivo* may be the same Passively transferred AA, however is inducible to a comparable extent in "tolerant' (anti-CD4-treated) and "non-tolerant' (BOM22-treated) rats, excluding active suppression of arthritogenic cells in the periphery as the mechanism of this tolerance Proliferative responses against SCW, *M tuberculosis* or the 65-kDa heat-shock protein were present in the BOM22-treated group (stimulation induces 25, 14 and 10 respectively), but absent in the anti-CD4-treated group (stimulation induces 5, 2 and 2 respectively) stressing the relation between arthritis and specific T cell responses

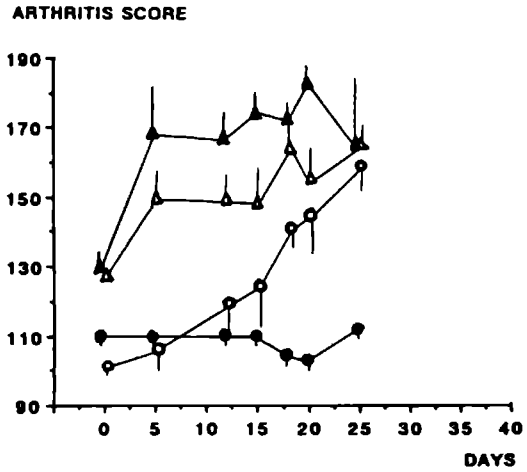

Figure 2 Rats were injected with 4 mg mAb (W3/25 = anti CD4) (●) or BOM22 (subclass control) (▲) at days 0 and 3 and an arthritogenic dose of SCW at day 0 At day 62 (= day 0 in this figure) rats were injected with 1 mg *M tuberculosis* in oil (AA) (●▲) or with *M tuberculosis*-specific T cells (passive transfer of AA) (○ △) Arthritis score is expressed as the sum of thickness of both hindpaws (× 0 1 mm) Each group consisted of three rats

3.4 Specificity of SCW plus anti-CD4-induced tolerance

To investigate whether the observed immunological non-responsiveness was for bacterial (arthritogenic) antigens only, we immunized rats which received mAb at days 0 and

Table 2 Influence of treatment with SCW and W3/25 or BOM22 on the induction of DTH reactions in the ear[a]

Treatment			DTH[b] R/L ratio of ear thickness[c]		
SCW	mAb	aBSA priming	24 h	48 h	
Day 0	W3/35, Days 0 and 3	Day 80	1 51 ± 0 03	1 61 ± 0 02	
Day 0	BOM21, Days 0 and 3	Day 80	1 53 ± 0 02	1 54 ± 0 02	
–	–	–	1 02 ± 0 01	1 01 ± 0 02	

a) The values represent the mean ± SD from groups of four rats
b) A DTH reaction was elicited by injection of 10 µg aBSA in the right ear of rats The left ear was injected with PBS
c) Values are obtained by dividing the measured ear thickness of the right (R) by the left (L)

3 and SCW at day 0 at day 80 with aBSA and tested for cell mediated immunity *in vivo* 15 days after immunization The response to aBSA as measured by a DTH reaction was similar in rats which were injected with SCW plus anti CD4 (tolerant) and in rats injected with BOM22 plus SCW (responsive) (Table 2) suggesting that the nonresponsiveness resulting in resistance to SCW arthritis and AA is not a nonspecific phenomenon

4 Discussion

There is considerable evidence suggesting a role for T lymphocytes in the pathogenesis of polyarthritis induced by SCW or mycobacteria in oil (AA) in the rat [1–5 7 14 17 18 27] Furthermore the induction of resistance to bacterium induced arthritis by low dose tolerance due to administration of the 65 kDa bacterial common antigen (heat shock protein) [18 28] or cell wall preparations (unpublished observations) stress the importance of (specific) T cells For AA use of anti CD4 antibodies has demonstrated the dependence of the model on $CD4^+$ cells [11]

Here we present evidence that $CD4^+$ cells (predominantly T_h lymphocytes) are essential for the induction of SCW induced arthritis and are highly involved in the effector phase In CD4 depleted rats SCW induced arthritis was not inducible Treatment which started in an ongoing arthritis (day 13) induced a significant remission Interestingly in rats treated in a prophylactic protocol no arthritis occurred after day 19 whereas at that timepoint $CD4^+$ cells had returned to normal level and a large amount of SCW was still present in tissues and circulation [15 16 26 29 30] One explanation might be the possibility that SCW were unaccessible for the immune system (due to sequestering in phagocytes for instance) Consequently rats were reinjected with an arthritogenic dose of SCW or with one third thereof (nonarthritogenic in naive Lewis rats) at day 83 The subclass control group developed an arthritis with an accelerated onset and a severity comparable to the first SCW induced arthritis In addition the arthritogenic and the subarthritogenic dose of SCW induced a similar arthritis Thus a previous SCW induced arthritis primes for a subsequent SCW induced arthritis rather than protects which is the case with AA [28] Rats which received anti CD4 mAb together with SCW however were completely resistant to further attempts to induce SCW arthritis suggesting a state of tolerance to SCW This is similar to the situation in AA [11] where anti CD4 treatment plus mycobacteria both prevented the induction of arthritis and rendered the rats resistant to renewed induction of the disease

To investigate the mechanism of this tolerance we induced AA and we passively transferred AA 62 days after injection with SCW and mAb Subclass control treated rats developed both the active and the passive AA with the former displaying an accelerated onset Anti CD4 treated rats were resistant to active AA but developed the passively transferred form Thus the state of tolerance is not maintained by active peripheral suppression but by a mechanism which acts earlier in T cell development (possibly in the thymus)

That pretreatment with anti CD4 at the time of priming with SCW induces resistance to SCW arthritis and AA and at the time of priming with *M tuberculosis* induces resistance to AA and to arthritis induced by the non antigenic synthetic adjuvant CP20961 [31] (unpublished observations) suggests that in all three models the same (auto)antigens are involved

The mechanism by which anti CD4 together with SCW induces specific nonresponsiveness may be similar to tolerance induction to human gamma globulin (HGG) in mice [32] administration of HGG together with anti L3T4 induced a state of HGG specific tolerance which did not involve $CD8^+$ cells This may be explained as follows [33–35] T cells that are stimulated by antigen (signal 1) only become responsive if they also receive signal 2 otherwise they are tolerized Signal 2 can consist of a signal provided by the APC as a result of cross linking events of surface receptors Removal of $CD4^+$ cells or functional blocking of CD4 may result in a weaker contact between MHC antigen complex on the APC and the TcR/CD4 complex on the T cell thus interfering with the second signal [32 36] For this tolerance to be everlasting the tolerogen has to be present continuously [32] In this context one can imagine that the presence of certain (auto)antigens which are relevant for arthritis may be auxillary in the maintenance of tolerance

Considering the Lewis rat and its enhanced susceptibility to autoimmune diseases in general and more specifically to bacterium/adjuvant induced arthritides it is possible that in Lewis rats the natural state of tolerance to autoantigens is a delicate balance which is more easily disturbed than in other rat strains When the immune system of Lewis rats is strongly stimulated with bacterial and synthetic adjuvants this state of tolerance can be broken Control of the already existing autoreactive repertoire is lost and chronic poly arthritis results The specificity of the existing but normally controlled repertoire may determine the organ specificity of the disease When rats are treated with anti CD4 at the same time that tolerance is broken the cells to be activated miss a second signal and become tolerant as long as the stimulus remains present If the stimulus is an autoantigen breaking of tolerance by nonspecific mechanisms together with anti CD4 may induce permanent protection in individuals susceptible to autoreactivity

We thank Wil Allebes (University Transfusion Service Nijmegen) for providing BOM22 Caroline Hicks (Lilly Research Centre Ltd Windlesham GB) for W3/25 and Maarten Hazenberg (Department of Immunology Erasmus University Rotterdam) for culturing S pyogenes organisms In addition we thank Piet Spaan (Central Animal Laboratory) for animal care

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CHAPTER 3

Cross-reactivity to proteoglycans in bacterial arthritis: lack of evidence for in vivo role in induction of disease

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Cross-Reactivity to Proteoglycans in Bacterial Arthritis: Lack of Evidence for *in Vivo* Role in Induction of Disease

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Cross-reactivity between bacterial epitopes and cartilage components has been assumed to play a role in the pathology of bacterial-induced arthritis models In this study, we report prominent proteoglycan (PG) depletion in Safranin-O stained ankle joint sections from collagen-induced arthritic rats. In adjuvant arthritis and streptococcal cell wall-induced arthritis (SCW-A), however, only limited PG degradation was observed *In vitro*, PG fractions were able to stimulate T lymphocytes from these arthritic rats To investigate the contribution of cross-reactivity, Lewis rats were primed with SCW in Freund's incomplete adjuvant (SCW/FIA) This immunization protocol resulted in *in vitro* stimulatory responses to the SCW antigens and cartilage PG antigens, but not to joint inflammation per se Next, papain was injected intraarticularly to create a situation in which a large amount of potential cross-reactive cartilage epitopes are released Interestingly, no inflammatory reaction could be observed in the papain-injected joints of SCW/FIA-primed rats

These data suggest that cross-reactivity between bacterial epitopes and PG does not seem to be a key element in the onset of joint inflammation in bacterial-induced arthritis However, it cannot be ruled out that at later time points cross-reactivity will contribute to joint inflammation ‹ 1994 Academic Press, Inc

INTRODUCTION

Chronic polyarthritis can be induced in susceptible rat strains by either structures of the joint itself, like collagen type II (1), or by bacteria Two well-studied bacterial models are the adjuvant arthritis (AA), induced by an intradermal immunization of heat-killed mycobacteria suspended in Freund's incomplete adjuvant (FIA) (2), and the streptococcal cell wall-induced arthritis (SCW-A), which can be induced by a single intraperitoneal injection of a sterile, aqueous suspension of SCW (3) For AA and SCW-A, both polymorph and mononuclear phagocytic cells (3, 4) as well as T lymphocytes (5–7) play an important role in the development and maintenance of disease In addition, we showed that the susceptibility to SCW-A in the Lewis and F344 strains appears to be correlated with the ability to mount a marked T cell response against SCW material (8)

However, the exact pathogenesis of these bacterial-induced arthritis models is still unknown Several *in vitro* studies suggest that cross-reactivity between bacterial epitopes and cartilage may play a role in disease development (9–11) In addition to this, it is possible to induce arthritis in BALB/c mice by immunization with human fetal PG (12)

Interestingly, *in vivo* studies which do indicate that cross-reactivity may play an active part in disease development are very limited In this respect, two studies reported modulatory effects with PG fractions (11, 13) However, whether these observed effects may also support that cross-reactivity is the initiating factor leading to disease development remains to be elucidated (14)

Therefore, we performed this *in vivo* study to examine the relevance of *in vitro* cross-reactive responses against cartilage PG in the pathogenesis of bacterial arthritis First, we investigated by histological analysis of Safranin-O stained total knee joint sections, whether cartilage of the inflamed ankle joints was depleted of PG at different time points after arthritis induction Compared to the collagen-induced arthritis (CIA) only limited damage was found in the bacterial models, which is not compatible with a strong autoimmune component Moreover, we looked *in vitro* whether we were able to detect any stimulatory effects by PG fractions in lymphocytes from AA and SCW-A rats

Finally, we performed experiments in which the cystein protease papain was injected intraarticularly in SCW FIA-primed rats (instead of intraperitoneal model) Injection of papain resulted in a marked depletion of PG within 24 hr, thus providing high levels of potential cross-reactive PG epitopes to initiate inflammation However, signs of persisting inflammation were absent

Our data show that it is unlikely that the observed cross-reactive T cell responses between bacterial epitopes and PG are important mechanisms leading to

joint inflammation in SCW-bacterial arthritis However, whether these cross-reactive responses become more important during the chronic phase of the arthritis cannot be ruled out

MATERIALS AND METHODS

Rats

Female Lewis rats were originally obtained from the Zentral Institute for Versuchstierzucht (Hannover, Germany) and were bred in our own facilities The rats weighed between 140 and 170 g at the beginning of each experiment and were fed standard food and tap water *ad libitum*

Induction of Arthritis

AA was induced by an intradermal injection at the base of the tail with 100 μl of 10 mg/ml mycobacterium tuberculosis (M tb) (H37RA, Difco Laboratories, Inc , Detroit, MI) suspended in FIA (Difco Laboratories) Before injection the M tb was finely ground in a pestle and mortar and suspended evenly in FIA

SCW-A was induced by an intraperitoneal (ip) injection of a sterile, aqueous suspension of SCW in a dose of 10 μg muramic acid/g rat body wt In order to obtain this SCW material, *Streptococcus pyogenes* T12 organisms were cultured overnight in Todd–Hewitt broth Bacteria were kindly provided by Dr H Verheul (Organon International, Oss, The Netherlands) After mechanical disruption (Braun MSK Cell homogenizer), the material was treated with RNase, DNase, and trypsin as described (3) Cell walls were further isolated by differential centrifugation steps as described (15), and the resulting 10,000*g* pellet was used throughout the *in vivo* experiments, while the 100,000*g* pellet was used *in vitro* The 10,000*g* preparation contained 15% muramic acid (16)

Type II CIA was induced by an intradermal injection into the tail base of 2 mg of native bovine nasal septum type II collagen The type II collagen was prepared by standard procedures (17) and was prepared for injection at a concentration of 20 mg/ml in a 50 50 emulsion of 0 1 *M* acetic acid and FIA

Priming of Rats

Female Lewis rats were primed in both forepaws by a subcutaneous injection of arthritogenic SCW fragments or ovalbumin (Sigma Chemical Co , St Louis, MO) emulsified in FIA (100 μl per injection site) The respective doses of antigen were 1 mg/ml muramic acid or 1 mg/ml ovalbumin Ten days later, animals received a subcutaneous booster of 100 μl at each side

T Cell Proliferation Assay

In the arthritis experiments popliteal lymph node were removed aseptically 10 days after arthritis induction

After a single cell suspension was made the leukocytes were washed twice with RPMI (Flow Labs Inc , McLean, USA) Adherent cells were removed by incubating the cells in a plastic culture flask (Costar) in RPMI containing 10% fetal calf serum (FCS) at a cell density of 5×10^6/ml After 1 hr in a CO_2 incubator nonadherent cells were aspirated and pooled per group Next the cells were put into culture with a final density of 1×10^6/ml in 0 2-ml vol of RPMI + 10% FCS + 10 m*M* pyruvate + 20 m*M* glutamine + 5 μ*M* β-mercaptoethanol + 40 μg/ml gentamycin Cells were cultured in 96-well round-bottom plates (Costar) for 3 days with variable concentrations of different cartilage components, bacterial antigens (100,000*g* pellet of SCW or M tb), or control stimuli Subsequently, 37 kBq [^{3}H]thymidine was added per well and cells were harvested 18 hr later All cultures were done in triplicate and pools of popliteal lymph nodes of three different animals were always used

Stimuli for T Cell Proliferation in Vitro

The PG subfractions were prepared as described earlier (18, 19)

Peptidoglycans were prepared from arthritogenic SCW material using a formamide extraction Briefly, 400 mg lyophilized SCW peptidoglycan–polysaccharides was added to 100 ml formamide (J T Baker, Deventer, The Netherlands) and heated at approximately 170°C for 1 hr (20, 21) The mixture was then centrifuged at 30,000*g* for 30 min to pellet the peptidoglycan fraction This formamide extraction was repeated four times After the final extraction the peptidoglycan was washed three times with 0 9% NaCl, dialysed against distilled water, and finally lyophilized Peptidoglycan fractions always contained less than 2% rhamnose by weight as determined by the method of Z Dische (22)

The cesium chloride (CsCl) top fraction (protein-rich fraction of bovine cartilage PG) was kindly provided by Dr R Kuijer (Department of Orthopedics, Maastricht) Briefly, proteoglycans were separated in collagen type II and PG by guanidinium chloride extraction (23) The non-CII was further separated by density centrifugation (1 47 g/ml CsCl, 108,000*g*, 10°C, 50 hr) in a protein-rich (top) and a PG-rich (bottom) fraction

Con A (Flow Labs Inc) was used as a positive control

In Vivo Cartilage PG Depletion

Cartilage PG depletion of knee and ankle joint in SCW-primed rats was induced by intraarticular (ia) injection of 1% papain (Merck, Darmstadt, Germany) in 10 μl phosphate-buffered saline (PBS) into the right knee and ankle joint of each animal As a control, an equal volume of PBS was injected into the left knee and ankle joint

Measurement of Unilateral Joint Inflammation

The level of joint inflammation was measured by the ^{99m}Tc uptake method (24) Briefly, rats were anesthesized with pentobarbital (300 mg/kg) and 0 2 ml (7 4 MBq/ml) ^{99m}Tc was administered subcutaneously After 30 min the ^{99m}Tc uptake in the right and the left knee and ankle joint was measured by external gamma counting The uptake of this small radioisotope is a measure for local blood flow and edema The severity of inflammation is expressed as right over left ratios and all values exceeding 1 1 were assigned as inflammation Joint inflammation was also scored histologically, as described below

Histology

Knee and ankle joints were removed *in toto* and processed for histology as described (25) Seven-micromolar sections were stained with either hematoxylin and eosin (H&E) to determine the level of inflammation or with Safranin-O which stains glycosaminoglycans, diminished staining would therefore represent PG depletion Scoring of inflammation and PG depletion was done by two independent observers

RESULTS

Cartilage Destruction in Bacterial Arthritis Models

In order to examine the occurrence of cartilage PG depletion in bacterial arthritis, Safranin-O stained sections of ankle joints were analyzed at various phases of AA and SCW-A At least four animals per time point were sacrificed and both ankle joints were examined for PG depletion

AA developed in all Lewis rats with an onset around Day 13 Sections taken at Day 17 revealed massive inflammation in the synovial tissue, but hardly any detectable loss of proteoglycans in the cartilage layers In fact, the cartilage of the talus and tibia appeared normal and minimal signs of depletion were noted at the margins underneath developing pannus tissue (Fig 1a) At later time points (Days 28 and 34) extensive bone marrow inflammation and bone erosion was found, but the cartilage of the talus and tibia remained unaffected (Fig 1b) Distinct cartilage PG depletion was regularly noted in a restricted, central area between the tarsal bones, showing outgrowth of granulation-like tissue (Fig 1c)

In the SCW-A model all Lewis rats developed a chronic arthritis, with a gradual onset in the ankle joints around Day 5 Sections taken at Days 7 and 10 showed a remarkable absence of cartilage PG loss, comparable to the observations in AA (Fig 1d) At later time points (Day 17) the degree of inflammation varied considerably, but even in severe cases cartilage PG depletion was restricted to the central area of the tarsal bones and the margins underlying pannus overgrowth

For comparison, similar sections were taken from ankle joints of Lewis rats with collagen arthritis In our hands, 75% of the animals developed an arthritis around Day 16 and these positive rats were further examined at Days 23 and 30 Cartilage PG depletion was clearly present in large parts of the cartilage, including the talus and tibia, and destruction was more marked at Day 30 Consistent with an autoimmune attack at the collagen type II-containing cartilage, even complete loss of the undecalcified cartilage layer was often found (Fig 1e)

T Cell Response to Cartilage PG

To test whether any cross-reactive responses against cartilage were present in AA and SCW-A, a lymphocyte proliferation assay was performed on the popliteal lymph nodes 10 days after arthritis induction A summary of the *in vitro* stimulatory effects for the various antigens is shown in Table 1 T cells from AA rats displayed a clear response to the bacterial antigen M tb and a significant response to the PG fractions

Regarding the T cells from SCW-A rats, only a low but significant response was observed against the injected antigen Interestingly, the T cells responded better to the peptidoglycan than to the SCW material, suggesting that the main T cell epitopes of SCW are on the protein-rich fraction and not on the polysaccharide part of SCW Furthermore, we were able to detect significant responses against the chondrosarcoma extract and link protein and an additional reactivity to the PG binding region

Joint Inflammation Due to Cross-reactive Immunity

To prime for cross-reactive responses Lewis rats were immunized with emulsified SCW/FIA, a protocol which does not lead to arthritis development The level of immunity in either the SCW/FIA or the control group (OVA/FIA) was tested in a lymphocyte proliferation assay Both groups of animals showed high responses against the antigen to which they were immunized and only low responses to the control antigen In addition, the SCW-primed T cells did show a significant response against the protein-rich fraction of bovine cartilage PG (Table 2)

To examine the potential contribution of this cross-reactivity to arthritis, papain was injected at Day 27 in right knee and ankle joints to release cartilage PG epitopes Three days later, the level of inflammation was determined by the ^{99m}Tc uptake method No swelling or edema could be detected in either group The histology (H&E-stained sections) did confirm the ^{99m}Tc uptake data, since almost no inflammatory cells could be observed in both ankle and knee joints (Table 3) In addition, sections were stained with Safranin-O to ver-

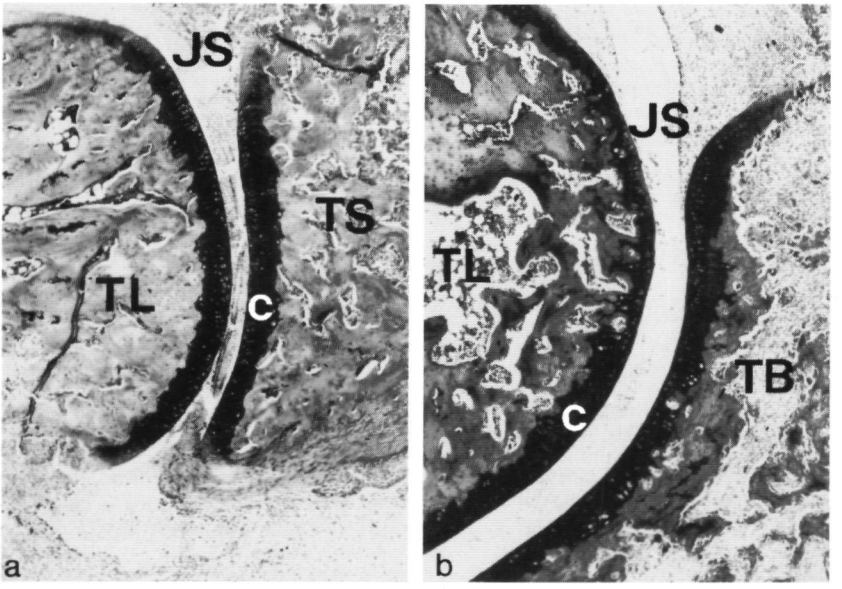

FIG. 1. Light microscopic analysis of cartilage PG depletion in Safranin O stained ankle joints in different arthritis models. (a) Adjuvant arthritis, Day 17. (b, c) Adjuvant arthritis, Day 28. (d) SCW-A, Day 17. (e) Type II collagen-induced arthritis, Day 23. (f) Negative control. JS, joint space; TL, talus; TS, tarsus; TB, tibia; C, cartilage.

ify whether the cartilage was depleted of PG due to the papain injections. In all Safranin-O stained sections of the right joints marked depletion of PG was observed.

DISCUSSION

Several *in vitro* studies do indicate that cross-reactivity between bacterial epitopes and cartilage may play a role (9–11); however, *in vivo* studies which support this hypothesis are very limited (11, 13). Therefore, we performed this *in vivo* study in both AA and SCW-A.

Histological analysis using Safranin-O stained sections showed only minimal PG depletion in both AA and SCW-A. The observation that some depletion was present in the areas of invading pannus are in agreement with an earlier report in AA (26). However, the finding that the cartilage of the talus and tibia was not depleted of PG at all has not been reported so far. To our knowledge only one other study investigated the cartilage of the talus in AA-treated rats by electron microscopy. In that study, it was shown that the proteinaceous outer-surface layer of the cartilage was disrupted (27), but whether the cartilage was depleted of PG was not investigated. Regarding the SCW-A, it also has been reported that extensive cartilage destruction does occur (28). However, major destruction was observed at later stages (Day 42), while only minimal destruction was observed at earlier time points.

As suspected, CIA showed prominent PG depletion and this was observed early on and throughout the entire ankle joint. These data do support earlier studies with H&E sections, which found that large parts of the cartilage became markedly eroded during the arthritis (1, 29). A likely explanation for this extensive PG depletion may be that in CIA, not only T cells play an important role (30), but also anti-CII MoAbs. These immunoglobulins attach to the cartilage surface and direct the inflammatory process toward this tissue. Of interest, CIA can be transferred with these MoAbs (31). Extensive cartilage destruction is also reported in the PG arthritis (32).

To conclude, our results of limited PG degradation during AA and SCW-A development do not fully support the hypothesis that arthritis may be initiated by structural mimicry between bacterial epitopes and cartilage.

The results of the *in vitro* study demonstrate that during induction of AA or SCW there is a recognition of PG components. The proliferative responses of the arthritic lymphocytes to the binding region were better in comparison to the whole chondrosarcoma extract. Regarding the T cells from SCW/FIA-primed mice and

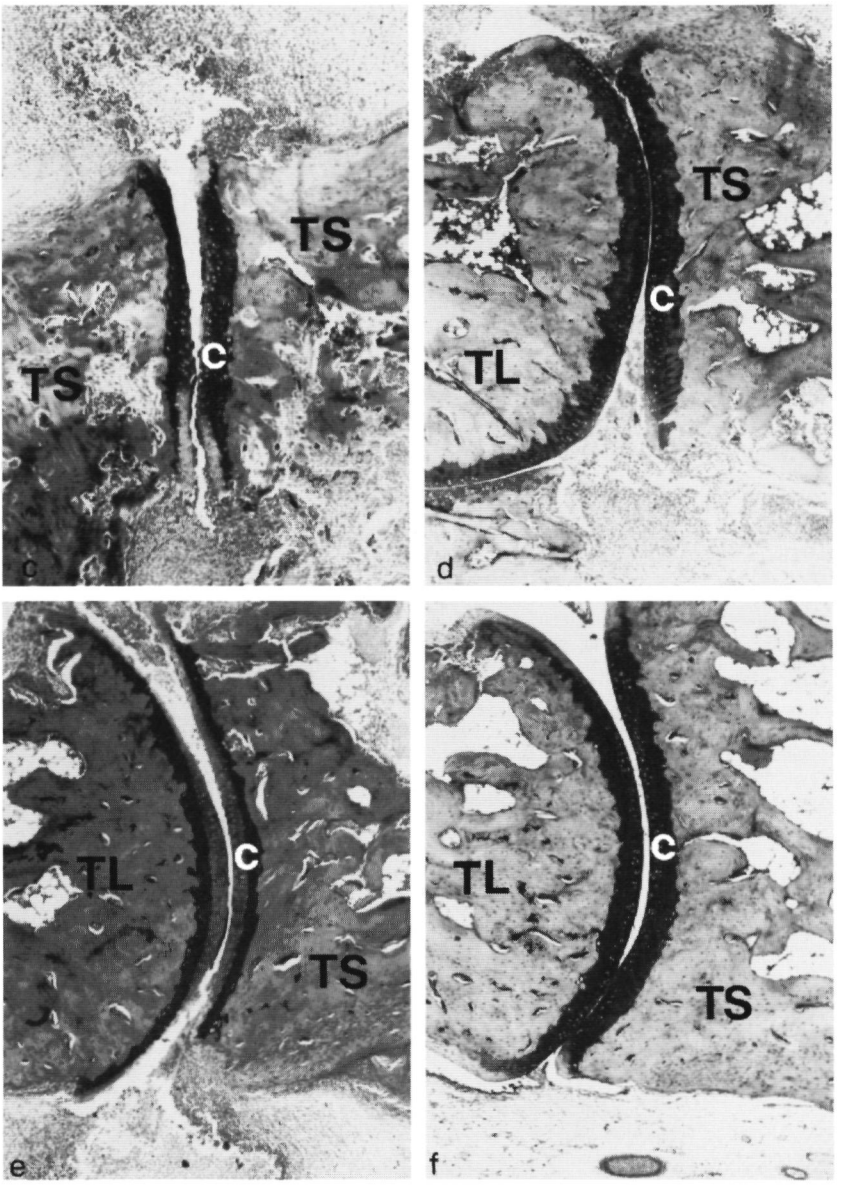

FIG. 1—*Continued*

TABLE 1

Proliferative Response of Lymph Node Cells 10 Days after Induction of AA or SCW-A

Stimulus	Concentration	Stimulation index AA	Stimulation index SCW-A
M tb.	30 μg/ml	8	—
SCW	30 μg/ml	—	2.3
Peptidoglycan	100 μg/ml	2.3	4
Link protein	1:10	1.3	2 2
Binding region	1·10	1.9	3.5
Chondrosarcoma	1:50	1.3	2.5
Ovalbumine	30 μg/ml	<1	<1
Con A	0 6 μg/ml	44	53
Control cpm		7436	6076

Note Proliferation was measured by [^{3}H]thymidine incorporation during the last 18 hr of a 96-hr culture with various bacterial and PG stimuli Proliferative responses are expressed as stimulation indices (amount of radioactivity incorporated due to stimulus X divided by that due to medium alone) The values represent the mean of triplicate cultures and the variation was always <10% Lymph nodes from three animals per group were pooled. Experiment shown represents one out of two experiments

T cells from AA rats, this has already been reported (10, 11), but in the SCW-A model in the Lewis rat this is the first report which shows cross-reactive responses against PG fractions. Some of the responses in AA were only minimal, but it cannot be ruled out that other components, such as keratan sulphate, chondroitin sulphate, or core protein, may induce higher responses (11). Furthermore, our data also indicate that intact native PG is antigenic, but PG subfractions are even more antigenic, which may suggest that degradation of PG during disease progression can expose sequestered or cryptic epitopes which are even more antigenic. The higher responses against peptidoglycans in the SCW-A may be explained by the fact that the degradation-resistant polysaccharide groups mask the antigenic determinants on the peptidoglycans. It also suggests that peptidoglycans contain most, if not all, T cell epitopes for SCW-primed T cells. That peptidoglycans are not capable of inducing chronic arthritis is due to lack of persistence. In this respect, the polysaccharide is of importance, since it protects the peptidoglycan from being rapidly degraded (14).

TABLE 2

Proliferative Response of Lymph Node Cells 23 Days after Immunization and Booster with SCW/FIA or OVA/FIA

Stimulus	μg/ml	Stimulation index SCW/FIA	Stimulation index OVA/FIA
SCW	6	29	3
OVA	30	2	8
CsCL top	30	6	2
Con A	0 6	168	62
Control cpm		901	1848

Note. Values represent the mean incorporation of [^{3}H]thymidine given as a stimulation index (amount of radioactivity incorporated due to stimulus X divided by that due to medium alone). Lymph nodes from two animals per group were pooled and the values represent the mean of triplicate cultures. Results shown represent one out of three experiments Various stimuli are used; SCW, streptococcal cell walls, OVA, ovalbumin, CsCl top, protein-rich fraction of bovine cartilage proteoglycan, Con A, concanavalin A.

TABLE 3

Effect of ia Injection of 1% Papain in Both Right Knee and Ankle Joint 30 Days after Immunization with SCW/FIA or OVA/FIA

Immunization	R/L ratio of ^{99m}Tc uptake Knee joint	R/L ratio of ^{99m}Tc uptake Ankle joint	Histological scores Right knee	Histological scores Right ankle
SCW/FIA	1 1 ± 0 08	1 06 ± 0 09	0 4 ± 0 3	0 2 ± 0.1
OVA/FIA	1 08 ± 0.1	1 07 ± 0 07	0 3 ± 0 2	0 2 ± 0 1

Note R L ratios of ^{99m}Tc uptake 3 days after intraarticular injection of 1% papain in SCW FIA or OVA/FIA immunized animals Values represent the mean ± SD from groups of eight animals After the ^{99m}Tc uptake assay the joints were removed and the sections stained with hematoxylin and eosin Inflammation was scored on coded slides, using a scale from 0 to 3 by two independent observers 0, no inflammation, 3, large mass of inflammation

To investigate the relevance of the *in vitro* cross-reactive responses in pathology, Lewis rats were immunized with SCW/FIA. Depletion of PG by an intraarticular injection of papain did not lead to joint inflammation. These data suggest that SCW-specific T cell responses, which *in vitro* cross-react with the protein-rich subfraction of PG, are not able to initiate an inflammation in our experimental circumstances. Together with the histological findings in AA and SCW-A, this indicates that it is unlikely that cross-reactivity to PG fragments is the initiating factor leading to joint inflammation in these models.

Several data do support the latter conclusion. First of all, it has been found in the SCW-A that a clear correlation exists between SCW dissemination and persistance in the joint and chronicity. Treatment with mutanolysin resulted in a decline of the chronic phase of arthritis (33). Mutanolysin degrades cell walls to small fragments, which show poor persistance in the joint and limited artritogenicity. In this respect SCW-A differs from AA. Furthermore, attempts to induce arthritis with subfractions of PG alone showed that these were never arthritogenic by itself (11). The finding that arthritis can be induced with human fetal PG (12) suggests that fetal PG are different in comparison to mature PG and that other epitopes are involved in PG and bacterial arthritis.

All together, we suggest that cross-reactive responses to PG fragments, which are present during the onset of disease, are not playing a pivotal role in the induction of arthritis. However, since arthritis can be modulated with PG fragments (11, 13), it appears that these cross-reactive responses are under control of similar regulatory mechanisms (34). An identical conclusion can be hypothesized from the 65-kDa experiments.

These data show that both AA and SCW-A can be modulated with 65 kDa (35, 36), and that responses against 65 kDa can be detected in animals with a bacterial-induced arthritis, but 65 kDa by itself is not arthritogenic (37) These results also indicate that responses against 65 kDa are not involved in the actual pathogenesis of bacterial arthritis, but probably act on regulatory mechanisms

It is important to realize that our study, aimed at the release of cross-reactive PG epitopes, was performed with papain and it cannot be excluded that endogenous enzymes like cathepsins or metalloproteinases may release other, more relevant epitopes Studies are now in progress to address this issue

In conclusion, our results imply that during the onset of disease, the observed *in vitro* cross-reactivity between bacterial epitopes and PG may not be the initiating factor leading to joint inflammation However, whether these cross-reactive responses may contribute to disease progression instead of disease induction remains a matter of debate

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CHAPTER 4

Cell migration studies in the adoptive transfer of adjuvant arthritis in the Lewis rat

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Cell migration studies in the adoptive transfer of adjuvant arthritis in the Lewis rat

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SUMMARY

Adjuvant arthritis (AA) can be induced in Lewis rats by a single injection of either heat-killed *Mycobacterium tuberculosis* or the lipoidal amine CP20961 Concanavalin A (Con A)-stimulated T cells isolated from AA rats are able to adoptively transfer the disease to naive syngeneic recipients It is unclear, however, whether these transferred cells traffic directly to the joint and initiate arthritis, or whether secondary host cells are responsible for activation of the disease In the current investigation, T cells labelled with the vital fluorescent dyes Hoechst H33342 and Zynaxis PKH26-G were used to adoptively transfer adjuvant disease to naive recipients At various stages of disease development sections of ankle joints, together with a range of soft tissues, were examined by fluorescence microscopy to determine the distribution of labelled donor cells in the recipients Intensely fluorescent lymphocytes were observed in the liver, spleen and lymph nodes within 24 hr of adoptive transfer Foci of such cells were clearly visible in the primary lymphoid tissues as late as 14 days after transfer However, close examination of both ankle joint sections and patellar fat pad cells throughout the time-course of the study failed to detect any labelled cells at the lesion site To develop these observations further we performed adoptive transfers to nude Lewis rats (rnu/rnu) and found that they were only moderately sensitive and developed, at best, a transient arthritis This observed difference could not be explained by a generalized lack of an inflammatory response, since we were able to elicit a zymosan peritonitis in the nude rats However, in nude Lewis rats a striking increase in adoptively transferred AA was obtained after reconstitution with 4×10^8 naive syngeneic spleen cells These combined observations suggest that a host-derived immune cell population is crucial for arthritis induction in the adoptive transfer system

INTRODUCTION

Rat adjuvant arthritis (AA) is a well-defined model of human rheumatoid arthritis that can be induced by immunization of Lewis rats either with *Mycobacterium tuberculosis* (Mtb) suspended in Freund's incomplete adjuvant (FIA),[1] or with the lipoidal amine CP20961 in paraffin oil [2] Fourteen days after induction, a progressive infiltrate of mononuclear cells is seen in the ankle joints, with concomitant early signs of bone remodelling Several studies have already shown the important role of T lymphocytes in this arthritis model AA cannot be induced in athymic (nude) rats,[3] treatment of rats with anti-T-lymphocyte antibodies prevents the induction of disease,[4,5] and, finally, the disease can be transferred to naive or irradiated syngeneic recipients with concanavalin A (Con A)-expanded T-cell lines[6] or clones [7]

The possibility that T lymphocytes can migrate specifically to the inflammatory site has been examined in several models recently In a dermal inflammation model, it has been demonstrated that a unique, small population of peritoneal exudate lymphocytes can migrate preferentially to cutaneous inflammatory sites[8-11] and reside there for long periods of time This contrasts with the kinetics of lymphocyte migration into the arthritic joint, which appears to be a major traffic site for all lymphocytes, although they appear to reside there for only a relatively short time [12] Furthermore, reports on the autoimmune disease experimental allergic encephalomyelitis (EAE), show that only a small percentage of transferred myelin basic protein (MBP)-reactive T cells accumulates in the central nervous system[13,14] and that non MBP-reactive lymphocytes ('bystander cells') can also play a role in mediating passively transferred EAE [15]

Recently, DeJoy *et al* [16] reported that in the adoptive transfer of AA some of the donor T cells (adjuvant sensitized) migrate to the synovium, where they may be responsible for disease induction However, subarthritogenic doses of affinity-purified anti-collagen IgG were co-injected to facilitate adoptive transfer of AA, thereby inducing a more rapid onset of disease Since this lesion appears to be complement dependent,[17] the co-injected anti-collagen IgG may stimulate the influx of inflammatory cells, including the transferred AA cells Thus it still

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remains to be demonstrated in the classical 'adoptive transfer system' which is primarily mediated by cellular immune mechanisms whether donor T lymphocytes are capable of specific migration to joints and subsequent initiation of arthritis

We therefore carried out adoptive transfer experiments of AA to naive recipients using either H33342-[8] or PKH26-[19] labelled Con A-stimulated T cells At various stages of disease development ankle joints and a range of soft tissues were examined by fluorescence microscopy to investigate whether any donor T lymphocytes could be detected Additionally adoptive transfer experiments were performed on immunocompromised recipients to determine whether a host-derived cell population plays an important role in the induction of disease in the adoptive transfer system

We were not able to detect any labelled cells in the joints suggesting that the cellular infiltrate at the lesion site is host derived Transfer experiments to nude Lewis recipients supported this hypothesis, since only moderate arthritis developed in these animals

MATERIALS AND METHODS

Rats

Female Lewis rats were originally obtained from the Zentral Institute fur Versuchstierzucht (Hannover Germany) and bred in our own facilities or were from Olac Bicester U K Female Lewis rnu rnu were obtained from Mollegaard (Skensved, Denmark) The rats weighed between 140 and 170 g at the beginning of each experiment and were fed standard food and tap water *ad libitum*

Adoptive transfer of adjuvant arthritis

The technique used was that described by Taurog *et al* [1] Briefly female inbred Lewis rats were injected intradermally at the base of the tail and the hind paws with 250 μg of Mtb (heat-killed human strains C, DT and PN Central Veterinary Laboratory Weybridge U K) The Mtb was finely ground in a pestle and mortar and suspended evenly in light paraffin oil at a concentration of 2 5 mg ml In case of the PKH26-GL labelling experiments donor cells originated from CP20961 (Pfizer Groton CT) (5 mg rat in 0 1 ml paraffin oil) primed animals Ten days after injection popliteal inguinal and subaortic lymph nodes and spleens were removed aseptically After a single-cell suspension was made the T lymphocytes were purified by passage over a nylon-wool column T cells were then stimulated with Con A (Flow Labs McLean VA) (3 μg ml) for 48 hr in RPMI supplemented with 10% fetal calf serum (FCS) 10 mM pyruvate 20 mM glutamine and 40 μg ml gentamycin (Flow Labs) at a concentration of $2\ 5 \times 10^6$ cells ml After incubation T lymphocytes were washed in phosphate-buffered saline (PBS) and either labelled or immediately injected intravenously into naive syngeneic recipients or irradiated recipients as stated (5×10^8 cells recipient) Arthritis developed within 5 days and was scored macroscopically by measuring hind paw thickness with an industrial micrometer or using the scoring system of Billingham *et al* [1]

Labelling of transferred cells

At the end of the *in vitro* Con A stimulation AA T cells were harvested and washed three times with PBS at room temperature These viable cells were then stained with either H33342 (Calbiochem La Jolla CA) or PKH26-GL (Zynaxis Malvern PA) dye The H33342 labelling was performed according to methods of Loeffler *et al* [18] Briefly lymphocytes were resuspended in a serum free Hanks balanced salt solution (HBSS) containing 1 μM H33342 at a concentration of 10^6 cells ml and incubated for 30 min in a 37 water bath with gentle agitation Cells were then washed twice in HBSS and viability and fluorescence were assessed by microscopic observation Labelled T cells were then used to transfer arthritis to naive animals

The PKH26-GL labelling was performed according to methods of Horan *et al* [19] Briefly 1×10^8 cells suspended in 5 ml of the kit (Zynaxis) diluent was added to a further 5 ml of diluent containing 4 μM PKH26 GL and incubated for 2 3 min at room temperature with gentle shaking The reaction was stopped by the addition of an equal volume of FCS for 5 min Twenty five millilitres of RPMI supplemented with 10 FCS was subsequently added and the cells were pelleted by centrifugation at 400 *g* for 10 min Cells were then washed twice once with RPMI supplemented with 10% FCS and once with PBS The fluorescence and viability were assessed by microscopy before intravenous (i v) injection into naive recipients

In vitro mitogen stimulation

In order to assess the effects of cell division on fluorescence intensity labelled donor T lymphocytes were maintained *in vitro* in the presence of Con A (3 μg ml) for 7 days The cultures were examined daily and the percentage labelling established by determining the ratio of fluorescent to non-fluorescent cells This was accomplished by counting the same haemocytometer field under both fluorescent and normal illumination

In vivo cell traffic experiments

Adoptive transfer experiments with H33342-labelled cells were performed four times and these data comprise the following days post-transfer 1 2 3 4 5 7 9 10 13 and 14 Adoptive transfer experiments with PKH26-GL-labelled cells were performed twice and comprise days 2 5 7 9 and 13 post-transfer At all the above time points groups of three recipients were killed and inguinal and popliteal lymph nodes thymus spleen liver and ankle joints were removed and immediately submerged in liquid nitrogen Multiple cryostat sections were cut and examined by fluorescence microscopy for the presence of labelled cells

In the lymph nodes at least three serial sections at different levels in the block were examined For the larger organs e g liver and spleen at least three sections were taken again at multiple levels in order to obtain as representative a sample as possible Three sections from at least two levels were examined in the joints Peripheral blood samples were collected from the dorsal aorta and leucocytes isolated by sedimentation in 0 5 methyl cellulose The percentage of labelled cells in the buffy coat was then determined as described above

Patellar fat pad cells

At days 2 5 7 and 9 two recipients were killed and the patellar fat pads removed Single-cell suspensions were made by enzymatic digestion using the method described by Dejoy *et al* Briefly the patellar fat pads were digested in serum-free RPMI supplemented with 0 5 mg ml collagenase 0 15 mg ml DNase and 0 05 mg ml hyaluronidase (Sigma Chemical Co , St Louis MO) After 90 min of gentle agitation at 37 the cell suspension

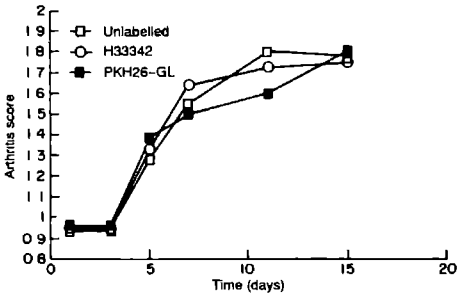

Figure 1 Comparison of the severity of AA after adoptive transfer with either labelled (H33342 or PKH26 GL) or non labelled T lymphocytes Arthritis was scored as the sum of paw thickness of both hindlegs (cm) Each group consisted of four animals

was centrifuged at 400 *g* and the cells washed with RPMI This suspension was filtered through a 60-μM Nytex filter (Tetko Elmsford, NY), and at least 100 cells examined by fluorescence microscopy

Irradiation of the recipients

Groups of four recipient Lewis rats were exposed to 2 5 or 5 Gy total body irradiation (TBI) The physical parameters of the X-irradiation were 18 MV, field size, 40 × 40 cm², dose rate 2 Gy min One hour after irradiation, arthritis was induced by adoptive transfer of Mtb-primed non-fluorochrome-labelled T cells

Induction of sterile peritonitis

Four rats from the control non-reconstituted nude and 5 Gy-irradiated groups were injected intraperitoneally (i p) with 18 ml of saline containing 1 % glycogen (Merck Darmstadt Germany) or 5 mg of zymosan (Sigma Chemical Co) Irradiation was performed either 2 or 5 days before induction of the peritonitis Sixteen hours after injection rats were killed and the peritoneal cavity washed once with 40 ml of saline The elicited peritoneal cells were washed, the total cell number determined using a Coulter counter, and differential leucocyte counts performed on Wright Giemsa-stained cytospin preparations At least 200 nucleated cells were identified as polymorphonuclear cells (PMN) or monocytes (Mo)

RESULTS

In vitro mitogen stimulation

H33342-labelled lymphocytes were cultured *in vitro* with Con A to investigate whether, during proliferation the dye was lost due to dilution Initially, all cells took up the label and, following 7 days of *in vitro* stimulation with Con A, 92% of viable cells remained fluorescent PKH26-GL has already been shown to be detectable *in vivo* after 121 days [22] Previous transfer experiments had shown that recipient animals developed macroscopic and histological signs of arthritis as early as 4 days following injection of donor cells These data therefore suggest that both dyes can be used effectively to determine the distribution of transferred cells during the period of lesion development

In vivo cell traffic experiments

Since the labelled cells were still clearly fluorescent following proliferation adoptive transfer experiments were performed with both dyes to ascertain whether either label affected the severity of the arthritis Labelled AA cells were transferred to naive recipients where it was found that neither label had any apparent affect on the incidence or severity of the arthritis in comparison with non-labelled cells (Fig 1) Time course experiments were performed subsequently using either the H33342 or the PKH26-GL dye which label the cell nucleus and cytoplasmic membrane respectively The results of the cell trafficking experiments were identical for both dyes and representative pictures of both dye are shown in Fig 2

At day 1 post-transfer the majority of fluorescent cells were localized in the liver and spleen Small foci of positive lymphocytes were observed in the popliteal and inguinal lymph nodes Approximately 5 % of the peripheral white cells were stained at this time this increased to a maximum of 90% on day 2 before falling to non-detectable levels at all subsequent time-points At days 2 and 3, the number of clearly labelled cells in the liver and spleen was seen to decrease whilst their frequency in the lymph nodes increased From days 4 to 14 foci of clearly labelled lymphocytes were still observed in the lymph nodes and spleen, but at the later time-points their numbers were reduced During the same period the liver and spleen developed a progressive autofluorescent appearance which was particularly marked in the animals which received H33342 labelled cells Macrophages sequestered the dyes and exhibited intense particulate cytoplasmic fluorescence Throughout the entire time course of the experiment no labelled cells were observed in the thymus

The examination of ankle joints at all time-points failed to reveal the presence of labelled cells at the lesion site, despite multiple sectioning at different levels of the block The absence of labelled cells in the joint was not due a lack of lesion development since in each adoptive transfer experiment macroscopic symptoms of arthritis were clearly observed

Patellar fat pad studies

Single-cell suspensions of patellar fat pads, prepared by enzymatic digestion, were examined by fluorescence microscopy No labelled cells could be detected at any time-point

Adoptive transfer to immunocompromised recipients

To determine whether a host-derived T-cell population is essential for arthritis induction, adoptive transfer experiments to immunocompromised (T-cell depleted) recipients were performed Rats that had received 2 5 Gy TBI developed a slightly less severe arthritis compared with controls However recipients that had received 5 Gy TBI developed a significantly milder arthritis ($P < 0.02$, Wilcoxon rank test) (Fig 3)

Adoptive transfer experiments were also performed with nude Lewis (rnu rnu) rats which are profoundly deficient in mature T lymphocytes [23] These experiments resulted in the initial development of a mild arthritis which failed to progress ($P < 0.01$ Wilcoxon rank test) compared with the control Lewis rats (Fig 4)

Subsequently, AA was transferred to nude recipients which had been reconstituted with 4×10^8 naive syngeneic spleen cells 1 day before the adoptive transfer Although no significant

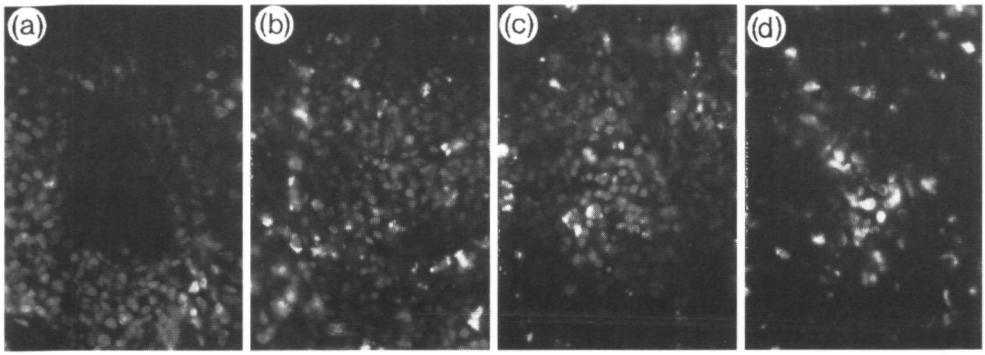

Figure 2. Analysis of the different tissues by fluorescence microscopy. (a) Liver, day 1 after transfer; (b) spleen, day 2 after transfer; (c) spleen, day 4 after transfer; (d) lymph node, day 14 after transfer. Magnification × 240.

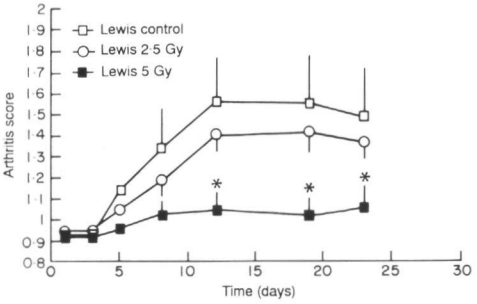

Figure 3. Adoptive transfer of AA to irradiated and non-irradiated recipients. Arthritis was scored as the sum of paw thickness of both hindlegs (cm). Each group consisted of four animals. Data were tested with the Wilcoxon rank test (*$P < 0.02$).

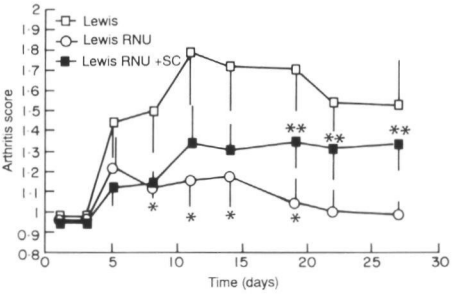

Figure 4. Adoptive transfer of AA to naive Lewis rats, nude Lewis (RNU) rats and nude Lewis recipients reconstituted with 4×10^8 naive spleen cells (SC) 1 day before transfer. Arthritis was measured using a calliper and the values represent the sum of the thickness of both hindpaws (cm). Each point represents 7–14 animals. Data were tested with the Wilcoxon rank test. Differences between the nudes and the naive Lewis rats were significant, *$P < 0.01$. Furthermore, the differences between the nudes and the reconstituted nudes were significant, **$P < 0.05$.

difference was observed at the onset of disease (nudes versus reconstituted nudes), it appeared that at later time-points the arthritis was significantly more severe and chronic ($P < 0.05$; Wilcoxon rank test) in the reconstituted nude recipients (Fig. 4).

It was noted that about 75% of the nude recipients, but not the reconstituted nude rats, died between day 22 and day 30 post-transfer. Macroscopic analysis did not show any signs of graft-versus-host disease.

Induction of sterile peritonitis

Adoptive transfer to T-cell depleted recipients resulted in a mild, non-progressive arthritis. A sterile peritonitis was therefore induced in control, irradiated and nude Lewis rats to determine whether such animals displayed a generalized inability to mount an immune response. In irradiated Lewis rats, it was found that the influx of inflammatory cells was markedly reduced compared to that of control rats. In contrast, the nude Lewis rats

Table 1. Peritoneal recruitment assay in immunocompromised animals

Recipient	*n*	Total cell no. $\times 10^7$ obtained from peritoneum	Identification of cells	
			PMN	Mo
Exp. 1				
Lewis	4	4·1 ± 2·3		
Lewis 5 Gy (day −2)	4	0·8 ± 0·2		
Lewis 5 Gy (day −5)	4	0·6 ± 0·4		
Exp. 2				
Lewis	4	8·4 ± 1·0	72 ± 2	28 ± 2
Lewis RNU	4	11·4 ± 0·7	60 ± 4	40 ± 4

Peritonitis was induced in Exp. 1 by injection of 1% glycogen and in Exp. 2 by 5 mg of zymosan.

showed the greatest response, recruiting more inflammatory cells to the peritoneum than any other group (Table 1)

DISCUSSION

Previous studies in rat AA have demonstrated the central role of T lymphocytes in this model [1 7] Furthermore, it has been reported[17] that a small number of T lymphocytes can be detected by immunocytochemical analysis of the knee synovium from rats undergoing adoptive transfer Whether the transferred T lymphocytes can migrate specifically to the joint remains a matter of debate Recently, DeJoy *et al* [16] have used an adoptive transfer system in which they were able to recover labelled cells from the knee synovium of recipients However, their system appeared to be complement dependent as anti collagen IgG was co-injected It thus remains to be demonstrated whether donor cells can be detected in the joint lesion when the disease is transferred by AA-specific lymphocytes in the absence of co-injected IgG In the current study, cell traffic experiments were undertaken to address this issue

Confirming earlier studies,[18 22 23] our data indicate that both dyes can be effectively used for *in vivo* cell trafficking experiments without affecting the incidence or severity of arthritis This suggests that the labelling does not alter the functional characteristics of these cells Perhaps surprisingly we were unable to detect any labelled cells in the ankle joints or patellar fat pads at any time-point in our adoptively transferred arthritis, this suggests that the cellular infiltrate within the joint lesion site is predominantly host derived It has been reported,[16] that T cells are present within inflamed synovium of adoptively transferred AA, and we have seen T cells in the later stages of adoptively transferred disease (unpublished observations) when lesions are well developed However, the appearance of T cells within lesion sites in the direct form of CP20961 in these Lewis rats, occurs much later than inflammatory macrophages, which enter the synovium and initiate the arthritis several days before T cells arrive (S C R Meacock and D R Brandon, manuscript submitted for publication) Our present data, therefore, suggest that the transferred T cells confer arthritogenicity to host cells This does not totally rule out the possibility that a few transferred T cells, undetected during our studies, entered putative lesion sites and set the stage for arthritis, they were certainly present in lymph nodes at day 14, but the overall lack of appearance of labelled cells at lesion sites infers initiation of the actual arthritis by host cells

The above findings are in agreement with the unpublished experiments of J D Taurog and colleagues (personal communication), who were also unable to detect any ^{111}In-labelled adoptively transferred cells in the arthritic joint This contrasts with adoptive transfer of EAE, where several investigators have detected small amounts of labelled cells at the lesion site [13 14] The difference between adoptively transferred EAE and our findings in AA may reflect the nature of the priming antigen In EAE the donor cells are primed against MBP, an antigen that is present in large amounts at the lesion site However, it remains unknown which antigens are recognized by Mtb- or CP20961-primed T cells in joint lesions in either direct or adoptively transferred arthritis

To support our findings further, adoptive transfer experiments were performed on T-cell depleted recipients Our data show that irradiation of recipients reduced their susceptibility to development of adoptively transferred AA in a dose-dependent manner This finding can be explained by a general immune dysfunction in these animals, as exemplified by their inability to recruit inflammatory cells to a non-specific peritoneal stimulus These observations concur with those of Taurog *et al* .[20] who have demonstrated that bone marrow reconstitution is essential in T-cell depleted recipients for initiation of arthritis

Adoptive transfer experiments were also conducted in nude (rnu/rnu) Lewis rats, which are reported to lack almost all mature T lymphocytes [24] The adjuvant disease transferred to these recipients was mild and non-progressive The lack of disease development could not be explained by a generalized immunodeficiency, since they were able to recruit inflammatory cells in response to an induced peritonitis Indeed, these nude rats were able to recruit even more macrophages and granulocytes to the peritoneum in comparison with normal Lewis rats This is perhaps not surprising, since it has been reported previously that nude rats do have higher numbers of circulating monocytes and granulocytes in peripheral blood [25] Small numbers of mature lymphocytes have been reported in nude rats,[26] this may explain the observation that these animals developed a mild, adoptively transferred arthritis Other potential mechanisms must not, however, be ruled out, and the role of the macrophage in adoptively transferred AA, for example, remains in question The high mortality incidence of the nudes at later time-points could be explained by the housing, since they were not maintained under specific pathogen-free conditions

Interestingly, the data obtained from experimental transfer of AA to nude rats contrast with adoptive transfer of EAE, since in this model the nude rats were as susceptible as the control recipients [2] Nevertheless, the latter data supports our present finding, that a host-derived T-cell population is essential for the development of arthritis by adoptive transfer Supporting evidence for this hypothesis was obtained by reconstituting the nude recipients with 4×10^6 naive syngeneic spleen cells These rats proceeded to develop a more severe arthritis than the non-reconstituted controls

The cellular interactions responsible for the arthritogenic effect of donor T lymphocytes are difficult to understand One plausible explanation may be that the passively transferred lymphocytes induce anti-idiotypic T cells in the host lymphoid tissue which are able to migrate specifically to the synovium These cells have been reported in vaccination experiments in EAE,[28] where anti-idiotypic cells were detected in the popliteal lymph nodes of rats that had been immunized with MBP-primed lymphocytes An alternative explanation is that transferred T cells may induce the activation of host macrophages which then migrate to the joint and initiate lesion development The recent observation that AA can be transferred by a macrophage-type of synoviocyte supports this hypothesis [29] Macrophage involvement would also explain why nude recipients were seen to develop a mild adoptively transferred AA in our experiments Further experiments are clearly required to test these hypotheses

In conclusion, our experimental data indicate that in the 'classical' adoptive transfer, which is primarily mediated by cellular immune mechanisms, a host-derived T lymphocyte population is crucial for arthritis development This is probably controlled within draining lymph nodes where the donor T lymphocytes initiate the host response

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CHAPTER 5

Regulation of resistance against adjuvant arthritis in the Fisher rat

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Regulation of resistance against adjuvant arthritis in the Fisher rat

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SUMMARY

Inbred female Lewis (LEW/N) rats develop a severe chronic arthritis in the adjuvant arthritis (AA) model, histocompatible Fisher (F344/N) rats are resistant and germ-free Fishers (GF F344) are again susceptible. In this study we show that the F344 rat can become susceptible to AA, using *Mycobacterium tuberculosis* (M.tb.) in the powerful adjuvant paraffin oil, instead of mineral oil (Freund's incomplete adjuvant (FIA)). This indicates that the F344 rat does not lack T effector cells. To examine further mechanisms responsible for suppression, we determined the level of plasma corticosterone in response to IL-1α in Lewis, F344 and GF F344 rats. IL-1α induced only low amounts of corticosterone in Lewis rats, but high amounts in both F344 and GF F344 rats. The GF F344 rats are susceptible to AA, but the severity of the disease is reduced compared with Lewis rats. This indicates that corticosterone may be an important mechanism to suppress disease development, but not the only mechanism. In addition we investigated whether T suppressor cells play a role in the resistance of the F344 strain. This was performed by pretreating the animals with the immunomodulating drugs cyclophosphamide (Cy) and cyclosporin A (CsA). We were unable to make the F344 rat susceptible to AA, indicating that active suppression does not play a role in the induction phase of arthritis. This finding is confirmed in adoptive transfer experiments of AA from Lewis to F344 rats. Our data suggest the lack of a strong pre-existing suppression in the F344 rats, and indicate that suppression is generated upon bacterial challenge. Whether suppression is overruled probably depends on the power of adjuvants used and potential control by corticosteroids.

Keywords adjuvant arthritis susceptibility corticosterone cyclophosphamide cyclosporin adoptive transfer

INTRODUCTION

Chronic erosive polyarthritis, resembling human rheumatoid arthritis, can be induced in susceptible rat strains by several bacteria. Two well studied models are the adjuvant arthritis (AA) induced by an intradermal immunization of heat-killed mycobacteria suspended in mineral oil [1] and the streptococcal cell wall (SCW)-induced arthritis, which can be induced by a single i.p. injection of a sterile, aqueous suspension of SCW [2]. For the AA as well as the SCW-A the susceptibility to arthritis seems to be regulated by the bacterial flora. In both models Lewis rats are susceptible, the histocompatible conventional Fisher (CV F344) resistant [3,4], and the germ-free Fisher (GF F344) again susceptible [5,6]. Some research has already been performed to explain this difference in susceptibility between the Lewis and F344 rat, but with a different underlying mechanism. We showed that the F344 rat was unable to mount a marked T cell response against SCW material, and that depletion of T suppressor cells *in vitro* led to a restoration of the T cell proliferative response [7]. This clearly demonstrated that active suppression can play an imporant role in the resistance of the F344 rat. Interestingly, however, depleting F344 rats *in vivo* of $OX8^+$ cells did not make this strain susceptible to exacerbations [8]. In addition, Hogervorst *et al.* [9] also found that in AA responsiveness to certain mycobacterial antigen epitopes could be related to susceptibility. However, in this study the non-responsiveness of the F344 appeared not to be due to antigen-specific suppression. On the other hand, Sternberg *et al.* showed *in vivo* that the Lewis strain has an impaired corticosterone response after an i.p. injection of SCW, and that this defect is located in the hypothalamic-pituitary-adrenal (HPA) axis [10]. This finding suggests that the ability to generate high levels of plasma corticosterone is the main mechanism to suppress disease development. However, the latter data are not necessarily in agreement with the observation that the GF F344 is susceptible.

We performed this *in vivo* study to gain more insight into the regulation of susceptibility in bacterial-arthritis models. Using

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the AA model we investigated which mechanisms play a role in the resistance of the F344 rat

First we investigated whether the resistance of the F344 strain can be overruled using more powerful adjuvants

In addition we examined whether the GF F344 like the Lewis also has an impaired corticosterone response We therefore compared the corticosterone responses to the inflammatory mediator IL 1α in both Lewis F344 and GF F344

Finally we investigated the presence of active suppression in CV F344 using pretreatment with low-dose cyclophosphamide (Cy) or low dose cyclosporin A (CsA) drugs proven to have anti-suppressor effects in AA [11 13] and experimental allergic encephalomyelitis (EAE) [14 15] Additionally we performed adoptive transfer experiments of AA using Lewis AA cells given to F344 rats to examine whether the F344 was able to suppress disease development when T effector cells were passively generated

MATERIALS AND METHODS

Rats

Female Lewis rats were originally obtained from the Zentral Institut fur Versuchstierzucht (Hannover Germany) and were bred in our own facilities Female F344 rats were also obtained from the Zentral Institut fur Versuchstierzucht Germ free (GF) F344 rats were originally obtained from Jackson Laboratories (Bar Harbor ME) and were bred in overpressure isolators in our own facilities The isolators and faeces were screened once every 2 weeks for bacterial contamination The rats weighed between 130 and 180 g at the beginning of each experiment and were fed standard food and tap water *ad libitum*

Induction of adjuvant arthritis

Arthritis was induced by an intradermal injection at the base of the tail with 100 μl of 10 mg ml *Mycobacterium tuberculosis* (M tb) (H37RA Difco Laboratories Inc Detroit MI) suspended in Freund's incomplete adjuvant (FIA Difco) or paraffin oil Arthritis was scored macroscopically by measuring hind paw thickness with an industrial micrometer One hundred per cent of the animals of the experimental groups developed an arthritis unless otherwise stated

In vivo corticosterone measurements

To diminish the stress caused by the experimental procedures each animal was handled daily by the experimentor for 15 min starting 1 week before decapitation One hour before decapitation animals were injected intraperitoneally with 1 μg of human recombinant IL 1α or saline Human recombinant IL 1α was a generous gift from Dr P T Lomedico (Roche Research Centre Nutley NJ) After decapitation blood was collected and plasma was stored at −20 C for subsequent hormone measurements Briefly this was carried out by extracting the plasma corticosterone with 7 5 ml of dichloromethane (Baker Deventer The Netherlands) The water phase was discarded and the dichloromethane phase was evaporated The residue was dissolved in 2 ml 0 2% ethylene-glycol water (EGW) and the concentration of corticosterone in the eluate was measured by a radioimmunoassay using an antiserum raised in sheep against a B 21 hemisuccinate bovine serum albumin (BSA) conjugate [16]

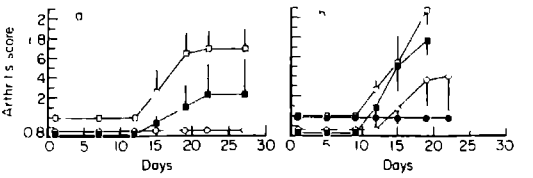

Fig 1 Effect of using different oil vehicles on the susceptibility of inbred Lewis F344 and germ free (GF) F344 rats for adjuvant arthritis Rats were immunized with 1 mg of *Mycobacterium tuberculosis* (M tb) in Freund's incomplete adjuvant (FIA) (a) or 1 mg of M tb in paraffin oil (b) (Lewis control received paraffin oil alone) Arthritis was measured using a calliper and the values represent the sum of the thickness of both hind paws ± s d (cm) Each group consisted of four animals □ Lewis ○ F344 ■ GF F344 ● Lewis control

Drug treatment

Cy (Endoxan-Asta 100 mg ASTA Pharma AG Frankfurt am Main Germany) was dissolved in sterile saline immediately before use and injected intravenously as a single dose of 10 25 or 50 mg kg body weight 2 days before arthritis induction by active immunization [11 12]

CsA (Sandimmune 100 mg ml Sandoz Pharma AG Basel Switzerland) was suspended in a 1 carboxy methyl cellulose (CMC) vehicle and given daily per os at a dose of 10 mg kg After 3 weeks AA was induced by active immunization as described above This protocol was proven to be successful in increasing the severity in Lewis rats (M Billingham Lilly Research Centre Windlesham UK personal communication)

Transfer of the adjuvant arthritis

In essence this transfer was performed according to the protocol reported by Taurog *et al* [17] and Billingham *et al* [18] Briefly draining lymph nodes (inguinal lumbar and popliteal) were removed aseptically from rats 10 days after immunization of M tb (125 μg suspended in 0 05 ml paraffin oil) in all four footpads and tail base After a single cell suspension was made the T lymphocytes were purified by incubating the cells on a nylon wool column (60 min 37 C) thereby removing the adherent cells T cells were then stimulated with concanavalin A (Con A 0 6 μg ml) for 48 h in RPMI supplemented with 10% fetal calf serum (FCS) 10 mM pyruvate 20 mM glutamin and 40 μg ml gentamycin (all Flow Labs Inc McLean VA) After 2 days cells were centrifuged down and suspended in PBS at a concentration of 5×10^8 cells ml and injected intravenously (1 ml) into naive non irradiated rats The arthritis was measured as described above for AA

RESULTS

Comparison of FIA with paraffin oil as vehicle for AA induction

AA was induced at day 0 in conventional (CV) Lewis CV F344 and in GF F344 using two different oil vehicles FIA or paraffin oil Figure 1a shows the results of FIA as oil vehicle in which all CV Lewis and 3 4 GF F344 are susceptible to AA and all CV F344 resistant However when paraffin oil was used instead of FIA both the CV Lewis as well as the GF F344 developed a more severe arthritis with an earlier onset (Fig 1b) Interestingly the CV F344 also became susceptible to AA with an onset around day 14 and an incidence of 100 Even a suboptimal

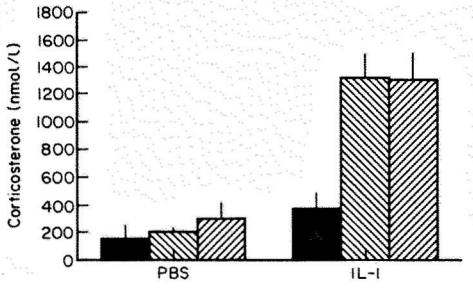

Fig. 2. Plasma corticosterone levels in Lewis (■), F344 (▨) and germ-free (GF) F344 (▩) rats. Rats of each strain were injected intraperitoneally with PBS control or 1 μg of human recombinant IL-1α. Corticosterone was determined in plasma collected 60 min post-injection. Data shown are means ± s.d. of four animals per group.

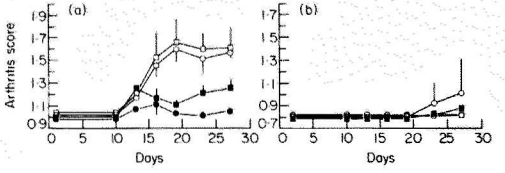

Fig. 3. Effect of cyclophosphamide on the development of arthritis in Lewis (a) and F344 (b) rats. Cyclophosphamide was given intravenously at three different single doses (mg/kg body weight) 2 days before arthritis induction. Arthritis was scored as the sum of the thickness of both hind-legs (cm). Each point represents the mean value ± s.d. of four animals. (a) □, Lewis control; ○, Lewis 10 mg/kg; ■, Lewis 25 mg/kg; ●, Lewis 50 mg/kg. (b) □, F344 control; ○, F344 10 mg/kg; ■, F344 25 mg/kg; ●, F344 50 mg/kg.

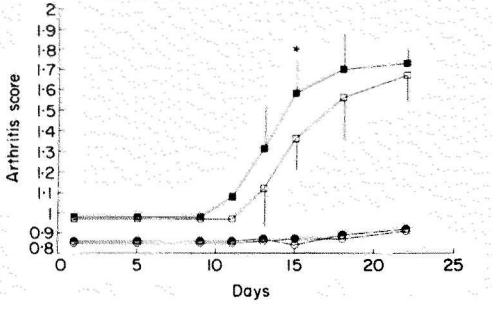

Fig. 4. Effect of oral cyclosporine A (CsA) pretreatment (10 mg/kg) on the development of adjuvant arthritis in Lewis and F344 rats. Daily treatment was started at day −22 and stopped at day 0, when arthritis was induced. Control rats received the vehicle carboxy methyl cellulose (CMC) alone. Arthritis was scored as the sum of paw thickness of both hind-legs ± s.d. (cm). Each point represents the average of eight animals. Data were tested with Student's *t*-test. $*P < 0.05$. □, Lewis; ■, Lewis CsA; ○, F344; ●, F344 CsA.

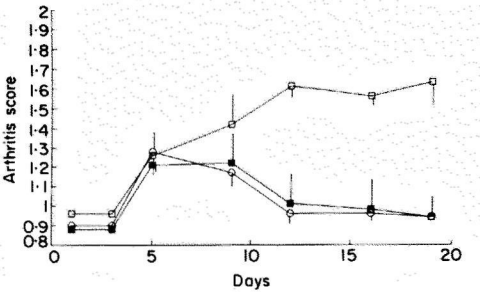

Fig. 5. Adoptive transfer of adjuvant arthritis to naive Lewis (□), F344 (○) and F344 germ-free (GF) (■) recipients. Arthritis was scored as the sum of paw thickness of both hind-legs ± s.d. (cm) using a calliper. Each point represents the average of 4–5 animals.

dose of M.tb., 0·1 mg/rat instead of 1 mg/rat, gave a 100% incidence in the CV F344 strain, though less severe (data not shown).

To rule out the possibility that the latter form of arthritis induction might be caused by the adjuvant alone, we tried to induce arthritis in CV Lewis rats with paraffin oil alone, but without success.

Corticosterone responses to IL-1α in CV Lewis, CV F344 and GF F344 rats

Since corticosterone is an important hormone because of its ability to suppress disease development, and can therefore have great influence on susceptibility to AA, we determined plasma corticosterone levels in response to the inflammatory mediator IL-1α in Lewis, F344 and GF F344 rats.

To exclude stress influences on the steroid levels, animals were handled daily for 6 days before IL-1 challenge. Intraperitoneal IL-1α, 1 h after injection, induced a marked plasma corticosterone response in CV F344 rats and only a minimal response in CV Lewis (Fig. 2). Interestingly, however, i.p. IL-1α induced an identical level of plasma corticosterone in the GF F344, compared with the CV F344.

Since handling of the GF F344 in the above experiment occurred outside the isolator, and susceptibility can change into resistance due to conventionalization [6,19,20], we checked whether the GF F344 rats were still susceptible to AA. Therefore, AA was induced in GF F344 rats, which were housed for 1 week in the conventional housing facilities. No difference in susceptibility was observed between the latter group and the GF F344, with a maximum arthritis score of 1·3 ± 0·3 and 1·4 ± 0·3, respectively.

Effect of drug pretreatment and susceptibility to an adoptive transfer

To study whether suppressor cells, already present or generated during the induction phase of the arthritis, are responsible for suppressing disease development, animals were pretreated with low doses of Cy or CsA, drugs proven to have anti-suppressor activity [21,22].

A single dose of Cy (10, 25 or 50 mg/kg body weight) was injected intravenously 2 days before arthritis induction. In the Lewis rat, Cy pretreatment inhibited arthritis development dose-dependently (Fig. 3a). In F344 rats none of the doses used

was able to make the F344 susceptible to AA, and even with a low dose of 10 mg/kg body weight, only one F344 (25%) developed an arthritis (Fig. 3b).

CsA (10 mg/kg body weight) was given orally before arthritis induction. In Lewis rats, CsA induced a more severe arthritis with an earlier onset, compared with the control rats (Fig. 4), which was significant at day 15 ($P < 0.05$; Student's *t*-test). Again no effect of CsA pretreatment was seen in CV F344.

It is still possible that suppressor cells were present, but are as sensitive to immunosuppressive agents as the arthritogenic cells. We therefore investigated whether it was possible to detect any suppressive activity by performing adoptive transfer experiments. As shown in Fig. 5, we were able to induce a chronic arthritis by adoptive transfer in the Lewis recipient with an onset at day 4. Transferring Lewis AA-specific T cells to CV F344 and GF F344 rats induced an identical onset and severity in the first days in CV Lewis, CV F344 and GF F344. At later time points arthritis declined more rapidly in both F344 strains, which may point to generation of suppression at that stage. However, it can not be ruled out that the latter phenomenon is caused by minor histocompatibility differences between Lewis and F344 rats.

DISCUSSION

In this study we investigated how the F344 rat may regulate its resistance to bacteria-induced arthritis. First, we examined whether a lack of T effector cells is responsible for the resistance of the F344 rat. Using a more potent adjuvant, M.tb. in paraffin oil, we were able to make the F344 rat susceptible, suggesting that the F344 rat does not lack T effector cells for arthritis development, data which are in agreement with arthritis susceptibility in GF F344 rats. Furthermore, this finding indicates that neither clonal deletion [23] nor clonal anergy [24] are the mechanisms by which the F344 regulates its resistance.

In addition, we show that Lewis rats injected with paraffin oil alone did not develop arthritis, suggesting that M.tb. is essential for arthritis induction. This is in contrast with the synthetic adjuvant model [25] or the adjuvant model reported by Kleinau *et al.* [26] and Cannon *et al.* [27], who were able to induce arthritis in DA rats with FIA alone.

Since a lack of T effector cells could not explain the observed differences between the Lewis and F344 strains, we investigated whether the susceptibility can be explained by impaired corticosterone responses to an inflammatory mediator. As can be seen in Fig. 2, the CV F344 rat, resistant to arthritis induction, had identical plasma corticosterone levels in response to IL-1α to the susceptible GF F344. The latter GF rats developed, however, an arthritis of lower severity compared with the one in Lewis rats (Fig. 1a). This suggests that the ability to generate high levels of corticosterone is an important mechanism of the F344 to suppress arthritis development, but leaving other mechanisms as important for the regulation of resistance.

Another likely candidate may be active suppression, especially since it was shown in *in vitro* studies in the SCW-induced arthritis model, that an impaired T cell proliferative response to the bacterial antigen could be restored by depleting $CD8^+$ T cells [7]. We were therefore interested to see if we could detect any *in vivo* suppressor activity in the CV F344 rat.

This was first examined by pretreating the animals with low dose Cy or low CsA, both drugs which have been shown *in vivo* to operate on the level of T cells, especially on T suppressor cells [21,22]. In the case of low dose Cy pretreatment in the Lewis strain, we only saw a decrease in arthritis severity, suggesting that even low dose Cy not only acts at the level of T suppressor cells but also at the level of T effector cells. This suppressive effect seen in Lewis can very well explain why we did not see any effect in the F344 strain. Earlier data with Cy pretreatment also show a dose-dependent effect. Low dose pretreatment enhanced the severity of AA in the WKA strain [11] and made the resistant Buffalo strain susceptible to AA [12], while a higher dose suppressed AA in the susceptible Holtzman and susceptible PVG/c strain [28]. Interestingly, low dose Cy also suppressed AA development in the susceptible Holtzman strain. This set of data is at variance with our results, and indicates that the net effect of Cy pretreatment may be strain-dependent. Furthermore, timing of administration is also of importance, since 20 mg/kg Cy given on days 0–2 after adjuvant prevented the development of arthritis in Sprague Dawley rats [29]. Therefore, treatments at later time points are not useful for studying the role of T suppressor cells in the F344. Because we were not able to detect any T suppressor activity in the F344 rat or increase in the severity of arthritis in the Lewis rat, we decided to test CsA pretreatment. The latter was proven to increase the severity in Lewis rats (M. Billingham, Lilly Research Centre, Windlesham, UK; personal communication) and therefore may be more effective for testing whether preexisting T suppressor cells are also responsible for the resistance of the F344 rat.

Indeed, CsA pretreatment was able to increase the severity in the Lewis strain, data which are in agreement with low-dose CsA pretreatment (2 or 4 mg/kg on alternate days for 22 days) in EAE [13]. However, the F344 rat still did not become susceptible to AA, indicating that either the level of suppression was too high to down-regulate, or the T suppressor activity, present during the induction phase of arthritis, does not play a role in the regulation of resistance in the F344 strain. Since CsA pretreatment primarily eliminates the preexisting T suppressor cells and hardly affects T suppressor cells generated during the onset of disease, the latter may still maintain the resistance of the F344. Some evidence for this up-regulation has been found by Kingston *et al.* (Lilly Research Centre, unpublished results), who found by phenotypic analysis that in AA in both Lewis and F344 rats $CD8^+$ cell numbers are increased, and that in the F344 strain the numbers of these cells remain elevated. This is in contrast to the Lewis strain, where $CD8^+$ cells decline as disease progresses.

Unfortunately CsA treatment at later time points, to eliminate these up-regulated T suppressor cells, is of limited use, since it has been frequently shown that this results in a suppression in several arthritis models [30–33].

Additional indications that the F344 rat has to up-regulate its suppressor activity were found in adoptive transfer experiments, since the F344 strain was susceptible in the transfer system. Because Lewis and F344 strains are reported to be syngeneic at the RT-1.A and RT-1.B region of the MHC region, and only differ with respect to minor histocompatibility antigens [34], we assumed that graft-*versus*-host reactions would not occur in these adoptive transfer experiments. Interestingly, the arthritis declined rapidly in both the CV and the GF F344 (Fig. 5), which can be explained by either the generation of suppres-

sion or by elimination of the transferred Lewis cells due to minor histocompatibility differences We have found several indications which do support the latter phenomenon e g no increase in the chronicity when the F344 recipient was pre-treated with low dose of Cy before the adoptive transfer and high responses in a mixed lymphocyte assay of T cells from an F344 recipient primed with Lewis T cells (manuscript in preparation)

Of course we considered studying the presence of active suppression in the F344 by *in vivo* treatment with OX8 (antisuppressor cytotoxic T) However several studies in AA [35 36] EAE [37 38] and an exacerbation study with SCW fragments in the F344 rat [8] have shown no effect of *in vivo* CD8 depletion indicating that this cell type plays no major role in the immunoregulation of AA In contrast in the SCW-A model in Lewis rats an increase in the severity and an earlier onset of disease were found (day 14 in the control group *versus* day 5 in the OX8 treated group) However the latter results are hard to interpret since this acute phase is considered to be T cell-independent [39] The fact that most studies do not show any effect of this OX8 treatment does not mean that T cells are not capable of maintaining resistance or down regulating inflammation It has been shown through analysis of murine Th cell lines that CD4 T cells can be classified into Th1 and Th2 lines based on the expression of IL-2 and interferon-gamma (IFN-γ) or IL 4 IL-5 IL-6 and IL-10 respectively [40] Because these two types of T helper cells synthesize lymphokines with profoundly different functions it is possible that *in vivo* these CD4 T cells may also be important regulators in the resistance of the F344 strain [41]

Taken together these data indicate that the F344 rat does not lack T effector cells for arthritis development and that the immune system of the F344 is also under control of a delicate balance of reactivity and suppression Furthermore we confirm that the ability to generate high levels of corticosterone may be an important mechanism of the F344 to suppress disease development However since we found identical levels in both CV and GF F344 rats this leaves other mechanisms like bacterial flora as important for the regulation of resistance to AA We cannot exclude the possibility that differences do exist in the kinetics of steroid levels in response to IL-1α Therefore time course experiments of steroid levels in cannulated F344 and GF F344 rats are now in progress after IL-1α challenge and during arthritis development Finally we have found clear evidence that preexisting active suppression does not play a role in the resistance of the F344 We assume that if any T suppressor mechanism does exist the F344 needs to up-regulate this upon bacterial challenge Unfortunately we were unable to prove the latter in adoptive transfer experiments since we found several indications that Lewis T cells are eliminated in the F344 due to minor histocompatibility antigens

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CHAPTER 6

Susceptibility to adjuvant arthritis: relative importance of adrenal activity and bacterial flora

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Susceptibility to adjuvant arthritis: relative importance of adrenal activity and bacterial flora

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SUMMARY

Previous studies on the regulation of bacterial-induced arthritis in rats have focused on endocrine aspects as well as differences in T cell immunity against bacterial epitopes We analysed the role of both adrenal activity and bacterial flora in determining susceptibility to bacterial-induced arthritis Outbred Wistar rats show a low incidence of adjuvant arthritis Moderate sensitivity to adjuvant arthritis was found in a selected stress resistant line of the Wistar rat, whereas no arthritis was found in a stress-susceptible Wistar line Plasma corticosterone responses after IL-1α exposure were however, identical in these two lines, excluding a direct correlation between susceptibility and corticosterone levels In line with previous findings in germ-free (GF) F344 rats, GF Wistars also appeared highly susceptible to arthritis We further analysed the corticosterone responses in GF and conventional (CV) rats Administration of IL-1α induced identical corticosterone responses in both CV and GF F344 rats In addition plasma corticosterone levels were measured around the time of onset of arthritis Whereas no rise was seen in the arthritis-resistant CV rats, a significant increase was observed from day 14 in GF rats, at the moment of onset of arthritis Although this corticosterone response was insufficient to prevent arthritis, it may have ameliorated disease expression in the GF F344 rats Our data indicate that the bacterial flora, and therefore T cell tolerance, is of prime importance in determining susceptibility, whereas the activity of the hypothalamus pituitary adrenal (HPA) axis may modulate disease severity

Keywords adjuvant arthritis susceptibility corticosterone bacterial flora

INTRODUCTION

Chronic erosive polyarthritis like adjuvant arthritis (AA) [1] and streptococcal cell wall (SCW)-induced arthritis [2], can be induced in susceptible rat strains Several investigators have examined mechanisms which determine the susceptibility for this type of joint inflammation It was found that susceptibility is strain dependent and appears to be controlled by an autosomal dominant gene locus linked to the MHC [3,4]

Not only genetic factors but also factors like the bacterial flora seem to regulate susceptibility to AA For example, Lewis rats are highly susceptible, while the histocompatible F344 rat is resistant and the germ free (GF) F344 rat is again susceptible [5 6] We showed that the ability to generate tolerance determines susceptibility since only the susceptible Lewis rats were able to mount a specific T cell response against the injected bacteria [6 7] Furthermore, Wilder and co workers showed that the Lewis strain has a deficient response of hypothalamic corticotropin releasing hormone (CRH) to various stressful inflammatory stimuli, while the F344 strain responded in a more robust manner [8] As a result the Lewis strain showed a deficient response of plasma corticosterone to these stimuli [9] and it was postulated that this diminished response of the hypothalamus pituitary–adrenal (HPA) axis plays a role in determining the susceptibility in the Lewis and F344 strains

In contrast to the Lewis strain, F344 rats display aggressive and stressful behaviour Recently two pharmacogenetically selected Wistar lines have been described which seem to differ in their neurochemical and neurobiological organization behaviour the apomorphine susceptible (APO-SUS) and apomorphine unsusceptible (APO-UNSUS) lines [10] The APO-UNSUS rats exhibit a decreased general locomotor activity and are less reactive to stressful stimuli than the APO-SUS animals [10,11] Of interest, the APO-UNSUS rat showed enhanced susceptibility to experimental autoimmune encephalomyelitis (EAE) [11]

To examine the relative importance of the neuroendocrine and immune system, we compared the susceptibility to AA in the outbred conventional (CV) and GF Wistar rats, as well as in

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the APO-SUS and APO-UNSUS lines Moreover, we determined plasma levels of corticosterone after IL-1α injection or during arthritis in the selected Wistar lines and in CV and GF F344 rats

MATERIALS AND METHODS

Animals

Pharmacogenetically selected F14 offspring female Wistar rats (APO-SUS and APO-UNSUS) were obtained from the Department of Psychoneuropharmacology Selection was made on their gnawing response to the dopamine receptor agonist apomorphine as described by Cools *et al* [10] Rats with gnawing responses < 10/45 min were designated as apomorphine unsusceptible (APO-UNSUS), and rats with gnawing responses > 500 45 min were called apomorphine susceptible (APO SUS) Retention of genetic selection was tested in rats of the first litter of each generation At the age of 60 days these rats were injected with apomorphine and tested as described earlier [10] APO-UNSUS rats with the lowest scores and APO-SUS rats with the highest scores were selected and used for subsequent development of the next generation Rats of the second and third litter of each generation were used for experiments Female CV outbred Wistar rats were obtained from Harlan CPB (Zeist, The Netherlands) and female CV inbred F344 rats from the Zentral Institut fur Versuchtierzucht (Hannover, Germany) Female Lewis rats were bred in our own facilities and were originally obtained from the Zentral Institut fur Versuchtierzucht

Female GF F344 rats were originally obtained from Jackson Laboratories (Bar Harbor, ME), and female GF Wistar rats from the Zentral Institut fur Versuchtierzucht and both were bred in our own facilities (Central Animal Laboratory) in overpressure isolators The isolators and faeces were screened once every 2 weeks for bacterial contamination

The rats weighed between 140 and 180 g at the beginning of each experiment and were housed in rooms illuminated from 6 00 a m to 6 00 p m Standard food and tap water were given *ad libitum*

Induction of adjuvant arthritis

Arthritis was induced by an intradermal injection at the base of the tail with 100 μl of 10 mg ml *Mycobacterium tuberculosis* (H37RA Difco Labs, Detroit, MI) suspended in Freund's incomplete adjuvant (FIA, Difco) Arthritis was scored by measuring hind paw thickness with an industrial micrometer In addition, in some experiments we performed a visual scoring on both hind and fore feet on the basis of 0 – no signs of redness or swelling, 1 – mild swelling and redness 2 = severe swelling and redness

In vivo *cannulation experiments*

In order to determine plasma corticosterone levels in response to administration of IL-1α or during AA, blood was collected from freely moving rats by means of a chronic cannula Cannulation of the rats was performed according to the technique described by Steffens [12] with some minor modifications as described by Sweep *et al* [13] After cannulation, the animals were housed individually and handled daily by the experimenter to diminish stress caused by the experimental procedures

Animals were allowed to recover from the operation for at least 6 days At the start of the experiment, a polyethylene cannula was attached to the stainless steel tube on the skull of the head Two hours later time course experiments were started ($t = 0$ min) by injection of 1 μg of IL-1α intravenously Recombinant human IL-1α was a generous gift from Dr P T Lomedico (Roche Research Centre Nutley NJ) Control rats received saline alone At every time point 300 μl blood were collected for corticosterone measurements, and subsequently 300 μl of saline were returned to the animal Regarding the time course experiments in AA, blood withdrawal always started around 11 00 a m , to exclude differences in corticosterone levels due to the circadian rhythm

In vitro *corticosterone measurements*

Blood samples were collected in dry lithium-heparin tubes and centrifuged at 1500 *g* for 10 min at 4 C Plasma was separated and stored at −20 C until assayed

Corticosterone measurements were performed as described by Sweep *et al* [13] Briefly, plasma corticosterone was extracted with 7 5 ml of dichloromethane (Baker, Deventer, The Netherlands) The water phase was discarded and the dichloromethane phase was evaporated The residue was dissolved in 2 ml 0 2% ethylene glycol water (EGW), and the concentration of corticosterone in the eluate was measured by a radioimmunoassay using an antiserum raised in sheep against a B-21-hemisuccinate bovine serum albumin (BSA) conjugate

The sensitivity of the assay was 25 45 fmol tube The intra- and interassay coefficients of variation were 5% and 8%, respectively

Statistical analysis

In the arthritis susceptibility experiments, the hind paw thickness between the different groups was compared by the Wilcoxon rank sum test The χ^2 test with Yates correction was used to analyse the results of the visual scoring

All corticosterone data are presented as the mean ± s d of five to eight animals Comparisons between the corticosterone levels in the arthritis experiments were made by the ANOVA test with repeated measurements Only when the ANOVA revealed a significant difference between both groups were values at specific time points further evaluated by Student's *t*-test for unpaired observations

RESULTS

Susceptibility of the different Wistar rat lines for AA

AA was induced in the APO-SUS and APO-UNSUS Wistar rat lines, and the incidence and severity of AA was compared with observations in CV and GF Wistar rats All APO-SUS rats were resistant, while 10 out of 16 APO-UNSUS rats appeared to be susceptible for AA ($P = 0.0001$, χ^2 test) (Table 1) When we measured the paw thickness only a moderate increase was noted in the APO-UNSUS rats, with a late onset (Fig 1)

The susceptibility of the CV Wistar rats, compared with the APO-SUS and APO-UNSUS rats appeared to be intermediate (3 out of 14) On the other hand, almost all the GF Wistar rats were susceptible for AA, and developed a severe and chronic joint inflammation (Fig 1, * $P = 0.0001$ *versus* CV Wistar rats, Wilcoxon rank sum test)

Table 1. Clinical expression of adjuvant arthritis (AA) in the different Wistar rats after the induction of AA

	0	1	2
APO-SUS	16	–	–
APO-UNSUS	6	6	4
CV Wistar	11	3	–
GF Wistar	1	4	6

The severity of arthritis was scored visually on a scale of 0–2: 0 = no redness or swelling; 1 = mild swelling and redness; 2 = severe swelling and redness. The data shown are the number of animals belonging to each group, 20 days after induction of arthritis. The susceptibility to AA between apomorphine susceptible (APO-SUS) and apomorphine unsusceptible (APO-UNSUS) was significantly different ($P = 0{\cdot}0001$, χ^2 test).

Corticosterone levels after IL-1α injection in APO-SUS and APO-UNSUS rat lines

We analysed the effect of IL-1α injection on plasma corticosterone levels in both APO-SUS and APO-UNSUS rats. This proinflammatory cytokine is able to stimulate the HPA axis, resulting in an increased plasma concentration of the anti-inflammatory steroid hormone corticosterone.

Intravenous (i.v.) injection of 1 μg IL-1α in cannulated APO-SUS and APO-UNSUS rats resulted in an increase of plasma corticosterone levels at 30 min (Fig. 2). No significant differences could be detected between corticosterone levels in the APO-SUS and APO-UNSUS rats. Corticosterone levels in control animals, injected with saline alone, remained low throughout the entire experiment.

Corticosterone levels after AA induction in Wistar rat lines

We examined corticosterone levels after the induction of AA in cannulated APO-SUS and APO-UNSUS rats. As shown in Fig. 3, no significant differences could be detected in plasma corticosterone between these two different rat lines (ANOVA, days 15, 18, 21).

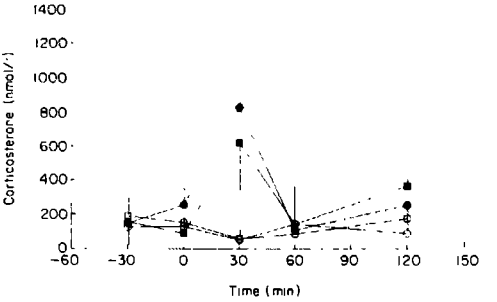

Fig. 2. Plasma corticosterone levels in apomorphine susceptible (APO-SUS) (●) and apomorphine unsusceptible (APO-UNSUS) (■) rats after i.v. injection of 1 μg of recombinant human IL-1α (closed symbols) at $t = 0$ min. Each time point represents the mean ± s.d. of eight animals. The control groups received saline alone (open symbols).

Susceptibility to AA in Lewis, CV F344 and GF F344 rats

It is known from previous studies that GF F344 rats are highly susceptible, whereas CV F344 are resistant to bacterial arthritis. We analysed the corticosterone response in these rats, both after IL-1 exposure and after induction of AA. Figure 4 shows the difference in AA susceptibility between the CV and GF F344 rats in this set of experiments. For comparison, data from Lewis rats are also given.

Corticosterone levels after IL-1α injection in F344 rats

The corticosterone response after i.v. injection of IL-1α was similar in CV and GF F344 rats (Fig. 5). Of interest, the peak levels were twice as high in F344 rats as in Wistar rats (Fig. 2). The control animals, injected with saline alone, showed no increase in plasma corticosterone.

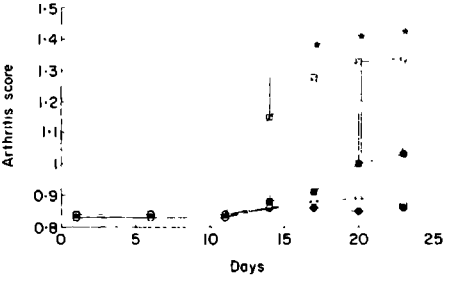

Fig. 1. Susceptibility of the same Wistar rats as shown in Table 1, but now scored by measuring the hind paw thickness with an industrial micrometer. Values represent the mean ± s.d. of 11–16 rats per group. The germfree (GF) Wistar rats (□) were significantly more susceptible than the conventional (CV) Wistar rats (○) * $P = 0{\cdot}0001$, Wilcoxon rank sum test. ●, Apomorphine susceptible (APO-SUS); ■, apomorphine unsusceptible (APO-UNSUS).

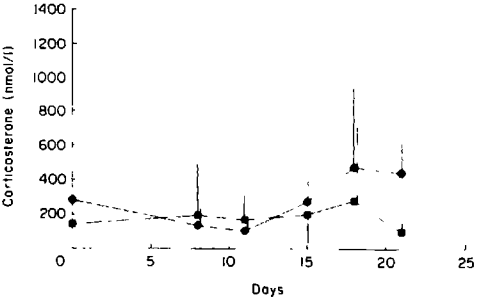

Fig. 3. Plasma corticosterone levels in apomorphine susceptible (APO-SUS) (●) and apomorphine unsusceptible (APO-UNSUS) (■) rats after induction of adjuvant arthritis (AA) at day 0. Blood samples were collected at 11:00 a.m. Values represent the mean ± s.d. of five animals per group. ANOVA (days 15, 18, 21) revealed no significant difference between both groups.

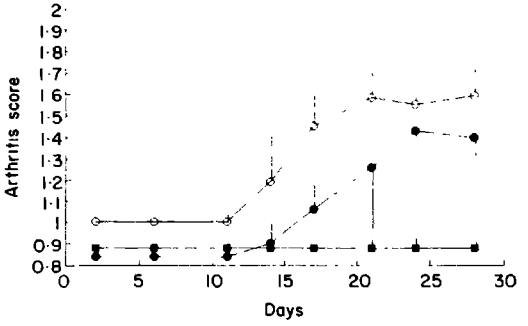

Fig. 4. Different susceptibility of Lewis (○), conventional (CV) F344 (■) and germ-free (GF) F344 (●) rats to the induction of adjuvant arthritis (AA). The arthritis was scored by measuring the hind paw thickness with an industrial micrometer. The values represent the mean ± s.d. of four animals per group

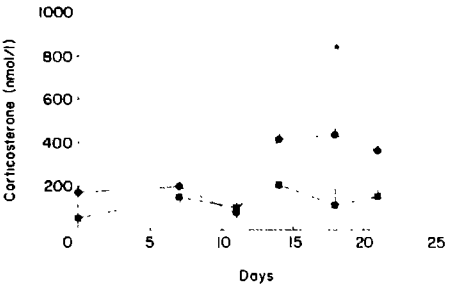

Fig. 6. Plasma corticosterone levels in conventional (CV) (■) and germ-free (GF) F344 (●) rats after the induction of adjuvant arthritis (AA). Blood samples were collected at 11:00 a.m. Values represent the mean ± s.d. of six animals per group. ANOVA revealed a statistically significant difference for both groups (days 14, 18, 21). day 18 * $P < 0.05$, Student's t-test.

Corticosterone levels after induction of AA in F344 rats

Finally, we analysed the course of corticosterone responses after immunization and during the onset of AA in F344 rats. Figure 6 shows that plasma corticosterone levels remained low in the resistant CV F344 rats during the whole period of observation. Compared with the CV F344, a significant increase (ANOVA $P < 0.01$, days 14, 18, 21) in corticosterone levels was observed during the onset of AA in the GF F344 rats (day 18; * $P < 0.05$, Student's t-test).

DISCUSSION

There is now increasing evidence that not only the genetic background, but also the bacterial flora and neuroendocrine system determine the overall immune response of an individual. In this study we analysed the relative importance of the activity of the HPA axis and the bacterial flora in determining susceptibility to AA.

The observation that the stress-susceptible APO-SUS Wistar rats were significantly less susceptible to AA than the stress-resistant APO-UNSUS rats is in agreement with recent findings in the EAE model [11], although the differences appeared to be more pronounced in the latter model. These findings suggest that different types of behavioural and neuroendocrinological responses to stress are correlated to different susceptibility to AA. Although we may not conclude that there is a causal relationship between stress and susceptibility to AA, several other data do indicate that stress can affect susceptibility. For instance, it has been observed that susceptibility to EAE can be partially suppressed by environmental stress [14]. Similarly, we observed that stress, induced by daily injection of a vehicle substance, may result in almost complete prevention of murine type II collagen arthritis ([15], manuscript in preparation). In addition, studies in patients with rheumatoid arthritis (RA) also demonstrated immunosuppressive effects of stress [16,17]. In contrast, other investigators emphasize that stress may selectively affect the T suppressor cell system in RA patients [18].

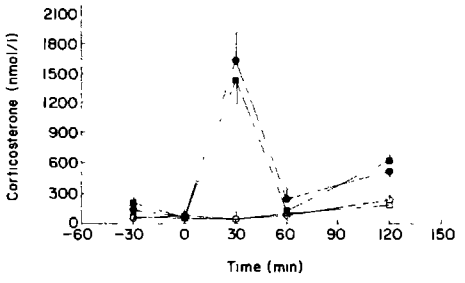

Fig. 5. Plasma corticosterone levels in conventional (CV) (■) and germ-free (GF) F344 (●) rats after the injection of 1 μg IL-1α intravenously at $t = 0$ min (closed symbols). Values represent the mean ± s.d. of six to eight animals per group. The control groups received saline alone (open symbols).

Interestingly, very recently differences were observed in T cell responsiveness between APO-SUS and APO-UNSUS rats (A. Kavelaars *et al.*, manuscript in preparation). It appeared that lymphocytes from APO-SUS rats had an increased mitogen reactivity and a higher number of natural killer (NK) and T suppressor/cytotoxic cells than lymphocytes from APO-UNSUS rats.

The fact that CV outbred Wistar rats show intermediate susceptibility [19], compared with the APO-SUS and APO-UNSUS rats, fits in with the fact that the APO-SUS and APO-UNSUS rats represent extremes in an unselected population of rats [10]. Furthermore, the observation that the GF Wistar rats were highly susceptible suggests that the bacterial flora seem to be extremely important in determining susceptibility to AA. This is in line with several previous studies, in which it was demonstrated that GF F344 rats were susceptible to bacterial-induced arthritis [5,6] and that conventionalization of these GF rats could dramatically moderate disease severity [6,20].

Analysis of corticosterone levels in response to administration of IL-1α showed that the genetic background can determine the activity of the HPA axis, since we observed a twice as high corticosterone response in the F344 rats compared with

the Wistar rats Interestingly all the time course experiments with IL 1α showed identical total corticosterone levels between the Wistar rat lines and between the CV and GF F344 strains This suggests that the activity of the HPA axis does not determine the susceptibility to AA in these different rats These data support our previous findings [21] but are in contrast with the findings in the susceptible CV Lewis rats [8 9] in which a clear defect of the HPA axis was observed Furthermore a recent study showed that RA patients may also have an abnormal HPA axis response to stress [22] On the other hand we can not totally exclude that differences in plasma steroid hormone levels may still determine susceptibility since we did not discriminate between free corticosterone and carrier bound corticosterone Regarding this Rots *et al* [23] also observed no differences in total corticosterone levels when the same Wistar rat lines were exposed to a novel environment as stress stimulus However when they compared free corticosterone as well as adrenocorticotrophic hormone levels they were then able to show a small increase in the HPA axis activity of the APO SUS rats

Analysis of the corticosterone levels after AA induction showed no significant differences between the APO SUS and APO UNSUS rats over a broad time span This finding parallels the only minor differences in arthritis severity between these two rat lines In the GF F344 rats however we were able to detect a significant increase in corticosterone levels after the onset of arthritis This increase may be due to the stress of the disease itself The latter phenomenon has also been observed during the onset of the clinical signs of FAE in the Lewis rat [24 25] On the other hand a recent report in AA shows increased levels of plasma corticosterone already at day 7 after immunization in Sprague Dawley rats [26] At that time point there were still no clinical signs of arthritis The difference might be explained by the fact that the HPA axis responsiveness during chronic stress is strain dependent

Finally no increase in corticosterone levels was observed in the resistant CV F344 rats which suggests that resistance of the F344 strain can not be explained by an increased HPA axis activity This indicates that the arthritogenic responses are probably controlled at another level A likely candidate is T cell tolerance especially in the light of our previous findings in which we were able to detect a correlation between T cell responses and susceptibility [6 7] On the other hand we can not exclude that the adrenal response in the C V F344 rats may only increase just after immunization (between days 0 and 7) and in this way directs the immune response to either suppression or reactivity However this is not very likely since an adrenalectomy study in the EAE model showed that corticosterone only had an important regulatory role just before the clinical onset of EAE in the susceptible Lewis strain [24] as well as in the resistant PVG strain [27]

In conclusion our data indicate that differences in behavioural and neuroendocrinological responses to stressors are correlated to differences in susceptibility to bacterial arthritis However the bacterial flora seem far more important in regulating susceptibility Furthermore the corticosterone data suggest that the HPA axis activity correlates with disease severity instead of being a mechanism to prevent disease Analysis of free corticosterone levels in the different susceptible rats and lymphocyte proliferation assays is now in progress These experiments will provide further insight into the relative importance of steroid hormone levels and T cell tolerance in susceptibility to bacterially induced arthritis

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CHAPTER 7

Role of ß2 integrins in the recruitment of phagocytic cells in joint inflammation in the rat

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Role of β2 Integrins in the Recruitment of Phagocytic Cells in Joint Inflammation in the Rat

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Adhesion molecules of the β2 family of integrins play an important role in adhesion and migration of leukocytes to inflammatory sites. Several *in vivo* studies indicate that not only monoclonal antibodies (mAbs) directed against the common β subunit (CD18) but also to the individual α subunits (CD11a, CD11b) can effectively inhibit different types of inflammation. In this study we report that in the adjuvant arthritis (AA) αCD11a, αCD11b, or even αCD18 treatment could not prevent disease development. Moreover, we examined the same mAbs in an acute nonspecific inflammation at different sites in the rat. We found that pretreatment with αCD11a or αCD11b could significantly block a zymosan peritonitis, but appeared to have no effect on a locally induced joint or dermal inflammation Interestingly, αCD18 treatment, which blocks the entire CD11/CD18 complex, was able to inhibit the influx of inflammatory cells in a peritonitis as well as in a joint and dermal inflammation These data not only indicate that the type of joint inflammation determines which adhesion molecules play a role in transendothelial migration, but also that involvement of the β2 integrins is highly site specific. © 1994 Academic Press Inc

INTRODUCTION

The process of adhesion and transendothelial migration of leukocytes plays an important role in the onset of inflammatory reactions Adhesion molecules involved in the attachment to the vascular endothelium comprise the integrins, a group of receptors containing several subfamilies (1) The CD11/CD18 complex belongs to the β2 subfamily of integrins and has three members with distinct α chains, CD11a, CD11b, and CD11c, and a common noncovalently bound identical β chain, CD18 CD11a/CD18 (LFA-1), CD11b/CD18 (CR3), and CD11c/CD18 (p150,95) are expressed on monocytes and granulocytes, whereas CD11a is also expressed on B and T lymphocytes (1, 2)

At present, monoclonal antibodies (mAbs) are available, which are directed specifically against CD18, CD11a, CD11b, or their ligand, the intercellular adhesion molecule-1 (ICAM-1) This makes it possible to examine the role of the different adhesion molecules in inflammatory models, like joint inflammation Lately, several *in vivo* studies have tested the effects of these mAbs in different types of joint inflammation Pretreatment with anti-CD18 (αCD18) could inhibit the influx of polymorphonuclear cells (PMN) to an IL-8-induced joint inflammation (3), whereas both αICAM-1 as well as αCD11a are able to suppress a peptidoglycan/muramyl dipeptide-induced arthritis (4) Furthermore, treatment with αCD18 not only inhibited the initial influx of inflammatory cells in the acute phase, but also resulted in an amelioration of the chronic phase of an antigen-induced arthritis (5)

Finally, it has been shown that adjuvant arthritis (AA) can be suppressed with αICAM-1 (6) and collagen-induced arthritis with either αICAM-1 or αCD11 (4) However, in the latter studies treatment was started early and also affected the generation of cell-mediated immunity against the injected antigen Therefore, it is hard to determine whether these mAbs selectively interfered with the migratory processes of inflammatory cells to the joint Of interest, the migration of PMNs to the AA joint seems to be mediated through CD11a, whereas T cells appear to use CD11a/CD11b-independent mechanisms (7)

The aim of the present study was twofold First, we investigated whether different adhesion molecules in the joint may be utilized for leukocyte migration, depending on the type of inflammation Second, we investigated whether the joint differs from other sites in the body with respect to the type of adhesion molecules used during inflammation We first analyzed whether the onset of AA can be prevented by blocking the migratory processes with mAbs against CD11a, CD11b, or all the β2 integrins This chronic joint disease can be induced in Lewis rats by immunization with heat-killed *Mycobacterium tuberculosis* in Freund's incomplete adjuvant (FIA) (8) Moreover, we examined the effects of pretreatment with the same mAbs in an acute, nonspecific inflammation For this purpose, zymosan was injected in the joint, peritoneum, or derm

It was found that none of the mAbs prevented the onset of AA Furthermore, pretreatment with αCD11a

or αCD11b only inhibited the migration of phagocytes to an inflamed peritoneum but not toward an inflamed joint or dermal inflammation In contrast, treatment with αCD18 was able to block effectively, independent of the site of inflammation

MATERIALS AND METHODS

Animals

Lewis rats were originally obtained from the Zentral Institut fur Versuchstierzucht (Hannover, FRG) and were bred in our own facilities Rats weighed 160–220 g at the start of the experiments and were fed tap water and standard chow *ad libitum*

mAb Production and Treatment

Hybridomas secreting αCD18 (WT-3, mouse IgG1), αCD11a (WT-1, mouse IgG2a) (9), both a kind gift from Dr M Miyasaka, or αCD11b (ED7, mouse IgG1) (10) were used to produce ascites fluid in BALB/C *rnu/rnu* mice by standard methods mAbs were purified using protein G–Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden) and stored at 4°C in saline (4 mg/ml) The control group received BOM22 (mouse IgG1 anti-human CD74), kindly provided by Dr W A Allebes (11) Rats were injected intravenously (i v) into the tail vein

Induction of AA

To induce AA, female Lewis rats were immunized intradermally at the base of the tail with 100 μl of 10 mg/ml *M tuberculosis* (H37RA, Difco Laboratories, Inc , Detroit, MI) suspended in FIA (Difco) The arthritis was scored macroscopically by measuring hind paw thickness with an industrial micrometer At the end of the experiment ankle joints were removed and the level of inflammation was determined by histology

Induction of Sterile Peritonitis

To induce sterile peritonitis, male or female Lewis rats were injected intraperitoneally (i p) with 5 mg zymosan (Sigma, St Louis, MO) dissolved in 2 or 4 ml of saline After 18 hr rats were sacrificed and the peritoneal cavity was washed once with 40 ml saline The isolated peritoneal cells were washed and counted in a Coulter counter Differential leukocyte counts of the peritoneal cells were determined morphologically using Wright Giemsa stained cytospin preparations At least 200 nucleated cells were counted per preparation

Induction of Unilateral Joint Inflammation

A nonspecific unilateral joint inflammation was induced by injecting either 0 2 or 0 6 mg zymosan in 60 μl phosphate-buffered saline (PBS) into the right knee joint of female Lewis rats As a control, 60 μl PBS was injected into the left knee joint of the same animal After 24 hr animals were sacrificed and the severity of arthritis was determined by histology

Induction of Intradermal Inflammation

A unilateral intradermal inflammation was induced by injecting 40 μg zymosan in 10 μl PBS into the pinna of the right ear in female Lewis rats As a control, 10 μl PBS was injected into the left ear After 24 hr the increase in ear thickness was determined using an industrial micrometer The severity of ear swelling was expressed as the ratio of the thickness in the right ear over the left (control) ear The rats were then sacrificed to determine the level of inflammation by histology

Histology

Knee or ankle joints and ears were removed and processed for histology as described (12) Paraffin sections (7 μm) were made and stained with hematoxylin and eosin (H&E) Scoring of inflammation was done by two independent observers on coded sections

mAb Treatment

In all the zymosan-induced inflammation experiments the animals were pretreated for 1 hr before in-

TABLE 1
Peritoneal Recruitment Assay

Treatment	*n*	Total cell number × 10^7 obtained from peritoneum	% Blockade	Identification of peritoneal cells PMN	Mo
Expt 1					
Control IgG	5	5 4 ± 1 3	—	ND	
αCD18		ND			
αCD11a	5	1 5 ± 0 6*	72	ND	
αCD11b	5	1 8 ± 0 8*	63	ND	
Expt 2					
Control IgG	10	10 2 ± 2 7		63 ± 3	37 ± 3
αCD18	10	1 8 ± 1 3*	82	66 ± 4	34 ± 4
αCD11a	10	6 6 ± 2 2**	35	63 ± 3	37 ± 3
αCD11b	10	6 0 ± 2 5**	41	69 ± 4	31 ± 4
Expt 3					
Control IgG	6	14 3 ± 2 2		ND	
αCD18	6	1 7 ± 0 3*	88	ND	
αCD11a	6	8 2 ± 1 3**	43	ND	
αCD11b	6	9 1 ± 3 3**	36	ND	

Note Effect of mAb pretreatment on leukocyte migration to the peritoneum of female (Experiment 1) and male (Experiment 2) Lewis rats 18 hr after 5 mg zymosan in 2 ml saline In Experiment 3 the peritonitis was induced in male Lewis rats with 5 mg zymosan in 4 ml of saline Blockade of the migration compared to the control is significant $^*P < 0.001$ and $^{**}P < 0.02$ (Wilcoxon rank sum test) In Experiment 2 differential leukocyte counts were performed morphologically using Wright giemsa stained slide preparations The ratio of polymorphonuclear (PMN) cells and mononuclear (Mo) cells was determined by counting at least 200 nucleated cells ND not done Presented are the mean values ± SD (*n* = number of animals per group) The majority of cells in the peritoneum are PMNs

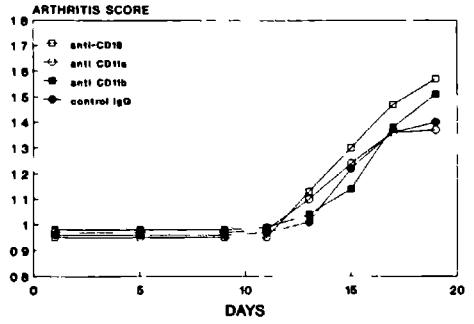

FIG 1 Effect of intravenous injections of 4 mg of αCD18, αCD11a, αCD11b and IgG1 control on the development of AA Treatment was performed every other day starting at Day 7 after immunization The arthritis score is expressed as the sum of both hind paws Each group consisted of six animals per group

duction of the inflammation Furthermore, all mAbs were injected into the tail vein at a dose of 2 mg, dissolved in 1 ml saline

Regarding the AA model, each animal was injected iv with 4 mg of each mAb (in 1 ml saline) every other day, starting at Day 7 after induction of the disease

FACS Analysis

In AA the treatment protocol was verified at Day 19 by collecting heparin blood of each animal for FACS analysis This FACS analysis was carried out using standard procedures Briefly, 100 μl heparin blood of each animal was incubated for 30 min at 4°C with either the control mAb or with the mAb used for *in vivo* treatment The samples were then washed in PBS–1% BSA and 0 1% sodium azide, and labeled at 4°C with FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse IgG (Dakopatts, Denmark) After 30 min the erythrocytes were lysed and the cells were washed with PBS–1% BSA and 0 1% sodium azide Finally 5000 cells from each sample were analyzed in a Coulter Epics flow cytometer

In addition, serum of each animal was collected and analyzed for the presence of free mAbs Therefore, cytospins of naive peritoneal cells were incubated for 1 hr with sera from the AA rats, which were injected with αCD18, αCD11a, αCD11b, or the control IgG1 These murine IgGs were then detected with rabbit anti-mouse IgG peroxidase (Dakopatts, Denmark)

RESULTS

Effect of mAbs in Zymosan-Induced Peritonitis

We first determined the inhibitory effects of a single treatment with 2 mg of either mAb in an acute peritonitis Blocking of either CD11a or CD11b appeared to be equally effective in inhibiting the influx of inflammatory cells (Table 1) In a relatively mild peritonitis, inhibition with both mAbs was even more prominent (Table 1, Experiment 1) Furthermore, pretreatment with the αCD18 mAb, which blocks the common β chain of the entire β2 integrin subfamily, almost completely blocked the influx of inflammatory cells into the peritoneum

Differential leukocyte counts showed that two-thirds of the infiltrating cells were PMN cells and one-third were mononuclear (Mo) cells No differences were observed in the PMN/Mo ratio between the different groups (Experiment 2)

Since we observed that these mAbs were effective in an acute peritonitis, similar dosages were used for modulation of joint inflammation

Effect of mAbs on Development of AA

In AA 2 mg of either mAb was injected every other day, starting 7 days after induction of disease (6 days before the first macroscopic signs) Since no effect was

TABLE 2

FACS Analysis on Circulating Phagocytes from Animals Treated with Different Anti-β2 Integrins during AA

Origin cells	Incubated with mAb	Mean fluorescent intensity and % blockade		
		PMNs	Lymphocytes	Mononuclear cells
Control IgG group	Control IgG	13 ± 1	8 ± 1	9 ± 1
	αCD18	214 ± 16	28 ± 3	540 ± 63
	αCD11a	170 + 11	37 ± 3	529 ± 51
	αCD11b	124 ± 7	11 + 1	235 ± 25
αCD18 group	Control IgG	181 ± 13 (93%)	17 ± 2 (81%)	564 ± 79 (95%)
	αCD18	194 ± 20	21 ± 3	596 ± 54
αCD11a group	Control IgG	104 ± 23 (81%)	15 ± 2 (100%)	488 + 71 (95%)
	αCD11a	128 ± 28	15 ± 3	512 ± 61
αCD11b group	Control IgG	169 ± 14 (97%)	11 ± 2 (92%)	184 ± 13 (97%)
	αCD11b	174 ± 15	12 ± 1	190 ± 21

Note Circulating blood cells from the different groups of animals were incubated with either the control isotype or the mAb used for *in vivo* treatment and the mean fluorescent intensity was determined on a FACS Each value represents the mean ± SD of six animals

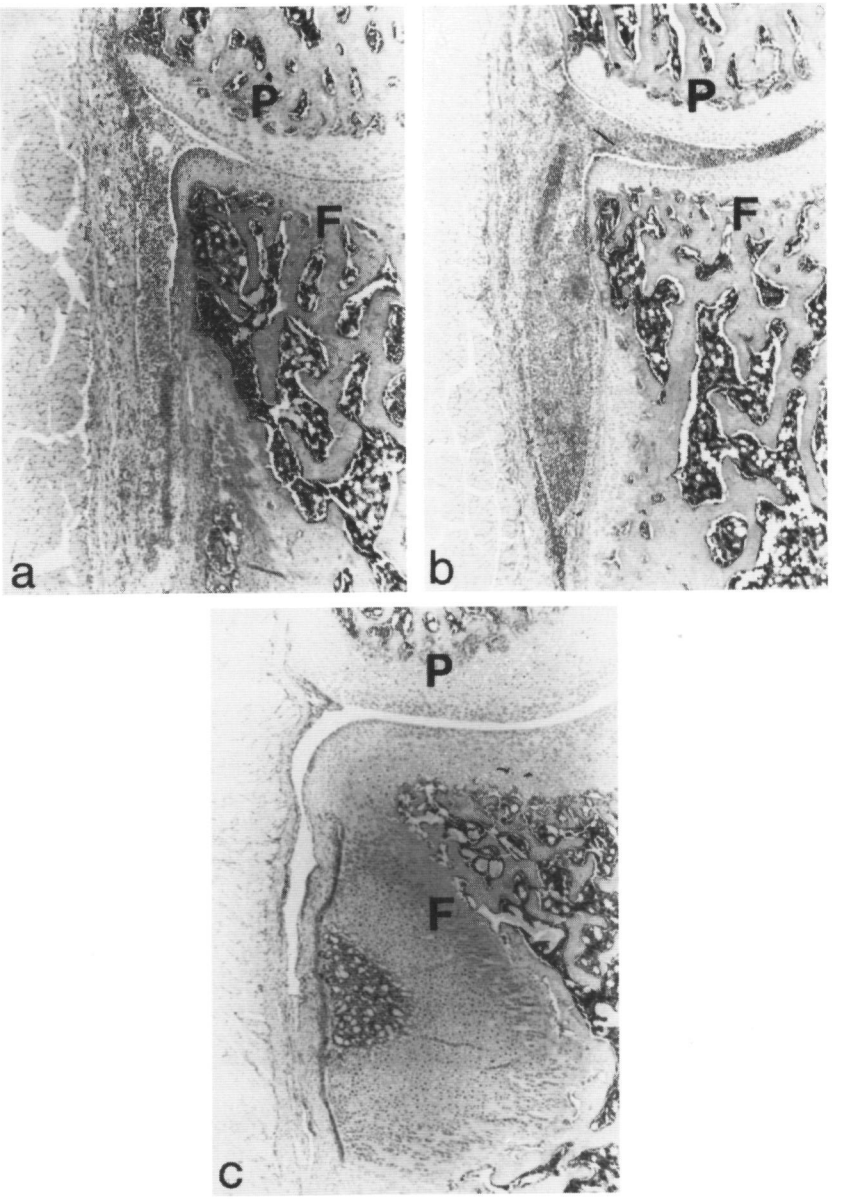

FIG. 2. Histology of rat knee joints 24 hr after 200 μg zymosan ia previously injected with either (a) αCD18 or (b) IgG1 control. Control joint was injected ia with PBS (c). Staining: hematoxylin & eosin. Original magnification: ×23. F, femur; P, patella.

observed with this protocol (data not shown), not even in the αCD18-treated animals, it was decided to increase the mAb dose to 4 mg However, even treatment with the high dose of either mAb did not suppress paw swelling in comparison with the control IgG1 (Fig 1) Moreover, histological analysis of the joints revealed no effect at all on the number of infiltrating cells, not even in the αCD18-treated animals (data not shown)

At the end of the experiment, 2 days after the last mAb injection, a FACS analysis was performed on the circulating leukocytes This was done in order to determine whether sufficient concentrations of either mAb had been administered This analysis showed that the binding of IgG to the various β2 integrins on the circulating phagocytes was still high and could hardly be increased by *ex vivo* incubation with the same mAbs (Table 2) Furthermore, Table 2 shows a high fluorescence intensity on PMNs and Mo cells, while incubation with αCD11a and αCD18 only showed a low fluorescence intensity on T cells In addition, a two-fold increase was found in the number of circulating phagocytes in the αCD18-treated animals, a phenomenon which was also observed in CD18-deficient patients (13)

Finally, immunocytochemistry showed that free mAbs were still present in sera of the different groups of animals, since peritoneal cells obtained from untreated rats, incubated with these sera, became positive for the various anti-β2 integrins (data not shown) Altogether these data indicate adequate mAb titers in the treated animals

Effect of mAbs on Locally Induced Zymosan Inflammation

Given the different effects of the anti-β2 integrin mAbs in a peritonitis, as compared with AA, it became of interest to investigate the role of the β2 integrins in inflammations at different sites, elicited with the same stimulus Zymosan was therefore injected in either the joint or derm after the animals were pretreated with αCD11a, αCD11b, or αCD18 Since 2 mg of either mAb was sufficient to inhibit the influx of phagocytes to the peritoneum, the same dose was used in the joint and derm

In a zymosan-induced joint inflammation, a dose-dependent increase of infiltrating cells in the control mAb-treated animals was observed and consisted predominantly of PMNs (Fig 2B) As seen in Table 3, αCD18 pretreatment blocked the influx of inflammatory cells in both the 0 2- and 0 6-mg zymosan-induced inflammation In contrast, with the peritonitis experiments, both αCD11a and αCD11b groups showed no decrease in the number of infiltrating cells Remarkably, αCD11b pretreatment even caused a minor, but not significant, increase in the level of inflammation at the highest dose

Injection of 40 μg of zymosan in the derm of the ear induced an inflammation which almost exclusively attracted PMNs (Fig 3B) Both αCD18 and αCD11a were able to reduce ear swelling, although αCD11a was less prominent (Table 4) αCD11b appeared to have no effect on the swelling (compared to the control mAb) Histological analysis of the H&E-stained sections showed that αCD18 pretreatment almost completely blocked the influx of PMNs In contrast, treatment with αCD11a alone tended to decrease the number of infiltrating cells, but this was variable and did not reach significance αCD11b pretreatment exhibited no effect at the histological level

DISCUSSION

Adhesion of inflammatory cells such as lymphocytes, granulocytes, and monocytes to the vascular endothelium is essential for transendothelial migration toward an inflamed site Several studies have already showed that *in vivo* phagocyte migration to the joint can be partially or completely blocked using specific mAbs raised against the β2 integrins or their ligand ICAM-1 (3–5)

Our peritonitis data are in agreement with earlier findings on anti-β2 integrin treatment of an inflamed peritoneum in mice (14, 15) A single study in the rat (10) showed a similar trend, although the net suppression of cell influx was less impressive In contrast, a recent report (16) observed a CD11/CD18-independent phase (24 hr) in a rabbit peritonitis model However, this study used different stimuli (peptone or *Escherichia coli*) to induce the peritonitis, suggesting that the regulation of migration might be stimulus dependent In addition, our data clearly indicate that these mAbs are effective for investigating the *in vivo* role of the β2 integrins in joint inflammation

TABLE 3
Histology Scores of Unilateral Zymosan Inflammation 24 hr after Intraarticular Injection

Treatment	Zymosan dose (mg)	Inflammation	% Blockade
Expt 1			
Control IgG	0 2	1 8 ± 0 3	
αCD18	0 2	0 7 ± 0 3*	60
αCD11a	0 2	1 6 ± 0 7	9
αCD11b	0 2	1 9 ± 0 5	−7
Expt 2			
Control IgG	0 6	2 4 ± 0 3	
αCD18	0 6	1 3 ± 0 4*	47
αCD11a	0 6	2 4 ± 0 4	0
αCD11b	0 6	2 8 ± 0 2	−16

Note Histological scores 24 hr after zymosan intraarticular Level of inflammation was scored on coded, hematoxylin and eosin stained slides 0, no inflammation, 3 large mass of inflammation *$P < 0.01$ (Wilcoxon rank sum test) Values represent the mean ± SD of six animals per group

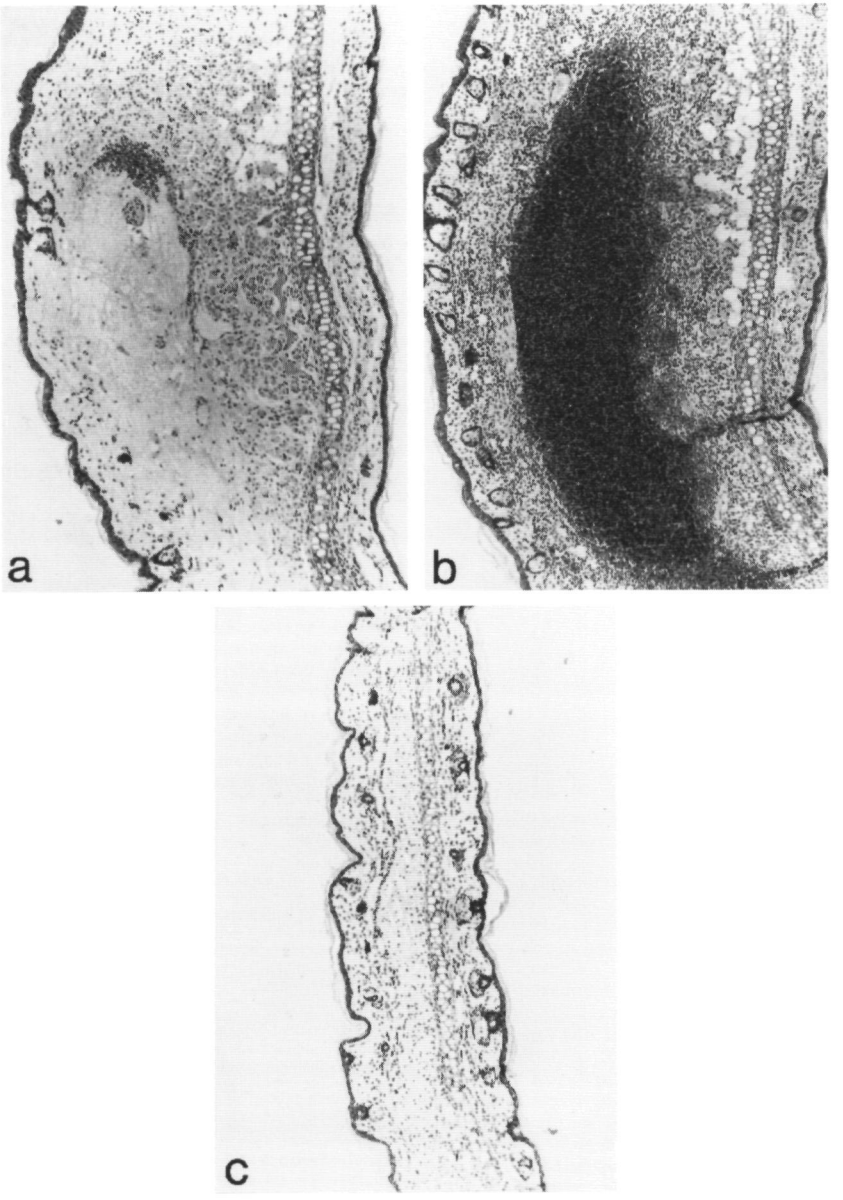

FIG. 3. Histology of rat ears 24 hr after 40 μg zymosan intradermally, previously injected with either (A) αCD18 or (B) control isotype. Control ear was injected with PBS (C). Staining: hematoxylin & eosin. Original magnification: ×90.

TABLE 4
R/L Ratios of Ear Thickness and Histology Scores 24 hr after Intradermal Zymosan Injection

Treatment	Swelling		Histological scores	
	R/L ratio	% Inhibition	Inflammation	% Blockade
Control IgG	1 83 ± 0 12		2 3 ± 0 7	
αCD18	1 45 ± 0 08*	46	0 3 ± 0 2*	89
αCD11a	1 66 ± 0 08**	20	1 8 ± 0 7	22
αCD11b	1 76 ± 0 03	8	2 1 ± 0 3	8

Note Effect of MAb pretreatment on ear thickness and leukocyte migration 24 hr after inducing an intradermal inflammation The severity of ear swelling is expressed as the ratio of the thickness in the right over that of the left ear *$P < 0.01$ and **$P < 0.05$ (Wilcoxon rank sum test) Level of inflammation was scored on coded hematoxylin and eosin stained slides 0 = no inflammation 3 = large mass of inflammation Values represent the mean ± SD of six animals per group

Earlier studies demonstrated that the β2 integrins play a role in Ag presentation (17) and cell–cell interactions between antigen-presenting cells and T cells (4, 6) To exclude this type of interference on generation of immunity in our adjuvant arthritis studies, we decided to start treatment with either mAb 7 days after induction of AA, at that time point AA can already be transferred with lymphocytes (18) Our protocol did not prevent AA, suggesting that the dominant migration of cells to arthritic joints is at least in part mediated via CD11a/CD11b-independent mechanisms This is in agreement with recent studies showing that a major portion of the PMNs and T cells use CD11a/CD11b-independent mechanisms during AA, αCD11a partially inhibited only the influx of PMNs (7), while αVLA-4 (α4β1) was able to selectively inhibit the influx of T cells (19) Furthermore, αICAM-1 treatment appeared to have at best minimal effects on the onset of AA in an adoptive transfer experiment (6)

In contrast, studies in other models, e g , insulin-dependent diabetes mellitus (20) and experimental autoimmune uveitis in mice (21) or autoimmune glomerulonephritis in rats (22), showed suppression of disease development with early anti-β2 treatment However, since late treatment showed no effects, interference with cell–cell interaction and the generation of autoreactive T lymphocytes may very well have accounted for these protective effects Interestingly, late treatment (Day 7) with αCD11b in rat EAE inhibited disease development (10) However, histological analysis showed no reduction in the number of infiltrating macrophages, suggesting that this treatment protocol may have influenced other phagocyte functions rather than migration Recent reports that αICAM-1 mAbs had no effect on EAE (23, 24) support the latter

Our FACS analysis data show that the expression of the CD11a and of course CD11b molecules was very low on circulating lymphocytes This suggests that the influx of T cells, which play such an important role in AA (18), is not suppressed and that the T cell-mediated regulation of phagocyte influx uses different integrins as compared with nonimmune inflammation Integrins like VLA-4 are expressed at higher levels on lymphocytes (25) and therefore may form a more promising target in inhibiting T cell-driven arthritis Interestingly, several *in vitro* studies show a prominent role for VLA-4 in the lymphocyte–rheumatoid synovium interactions (26–28)

Our results with αCD18 treatment in nonimmune inflammation are in agreement with earlier reports on αCD18 mAbs in the joint (3, 5) and other acute inflammatory models (29–35) Regarding the selective blocking of either CD11a or CD11b, variable results have been reported Slight inhibitory effects were found with αCD11a in the joint (4, 7) and derm (36) Recently, Mulligan *et al* (37) reported that a PMN-dependent inflammation could be effectively blocked with αCD11a, whereas an inflammation in which macrophages were the main effector cells appeared to be more CD11b dependent In our zymosan-induced joint and dermal inflammation, PMNs were the main infiltrating cells, yet we did not observe significant suppression with αCD11a Pretreatment with αCD11b had no effect in a dermal inflammation and even slightly enhanced a joint inflammation Interestingly, this latter effect has also been reported in a dermal inflammation model (36) In that study it was suggested that blocking of CD11b may prime or activate phagocytes for a more efficient response to C5a

In conclusion, the relative contribution of the various adhesion molecules seems site and stimulus specific, a phenomenon which has also been observed *in vitro* (38) Our data suggest that inhibition of one β2 integrin in the joint and derm is insufficient and might even facilitate the usage of other β2 integrins In the peritoneum, influx requires the combined expression of CD11a and CD11b since blocking one or the other is already sufficient to diminish the influx of PMNs and macrophages Whether these differences reflect low expression of adhesion molecules on peritoneal endothelium per se or local release of different cytokine balances remains to be investigated Future studies with mAbs directed against other integrins (VLA-4, CD11c) or combinations of these mAbs should help unravel the applicability to block particular joint inflammations

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CHAPTER 8

SUMMARY/SAMENVATTING

SUMMARY AND CONCLUDING REMARKS

Rheumatoid arthritis (RA) is a chronic and erosive joint inflammation of an unknown etiology. To facilitate research on this autoimmune disease, several animal models are available, which contribute to the elucidation of the pathogenesis of this joint inflammation. Two well studied bacterial arthritis models are the adjuvant arthritis (AA) and the streptococcal cell wall-induced arthritis (SCW-induced arthritis). For AA and SCW-induced arthritis both polymorph and mononuclear cells as well as T cells are essential for the development and maintenance of disease. However, the exact pathogenesis of these bacterial-induced arthritis models is still unknown. On the one side one can think of a specific T cell reaction against bacterial fragments, which are present in the joint, while on the other hand crossreactivity between bacterial epitopes and cartilage may play a role in disease development. Apart from the genetic background and the immune system some studies also suggest that the neuroendocrine system may be crucial in regulating the susceptibility to bacterial-induced arthritis. Furthermore, there is increasing evidence that both the neuroendocrine and immune system are able to communicate through overlapping regulatory mediators.

In this thesis we have studied the regulatory aspects of the immune and neuroendocrine system in bacterial arthritis models. First of all we were interested in whether crossreactivity had any in vivo relevance in the pathogenesis of these bacterial arthritis models. In addition, we wanted to gain more insight in the mechanisms which are important in regulating the susceptibility in different rat strains and lines. Finally, we investigated several ways to modulate arthritis with selective blocking or elimination of the inflammatory cells.

In the first study we confirm earlier findings, that T cells are important in these bacterial-induced arthritis models. Treatment with anti-CD4 in a prophylactic protocol resulted in virtual absence of SCW-induced arthritis, while on an established disease only a minimal suppression was observed. Furthermore, we found that treatment with anti-CD4 plus SCW induces a long-term resistance to both SCW-induced arthritis and AA. This indicates that induction of T cell tolerance can be a powerful mechanism to maintain prolonged resistance against this type of arthritis (Chapter 2) Recently, clinical trials have been started with chimeric monoclonal anti-CD4 antibodies. These studies show that treatment with these antibodies were able to decrease the number of circulating T cells, while no significant adverse effects like e.g. infectious complications were observed. Regarding the efficacy of this treatment the data are not unequivocal, but do indicate that the type of antibody, the dosage and treatment regimen are crucial factors in determining the clinical outcome.

In SCW-induced arthritis and AA the exact pathogenesis is still unknown. Several in vitro studies suggest that cross-reactivity between bacterial epitopes and cartilage may be involved in disease development. However, in vivo indications for this hypothesis are limited. Sofar, only immuno-modulatory effects of cartilage proteoglycan (PG) fractions have been desribed. Therefore, we studied the relevance of the in vitro cross-reactive responses against cartilage PG. First, we examined Safranin-O stained ankle sections, and found only minimal PG depletion in both AA and SCW-induced arthritis. These histological observations do not fully support the hypothesis that arthritis may be initiated by released PG's and structural mimicry between bacterial epitopes and cartilage. Interestingly, T lymphocytes of these arthritic rats were able to respond to the PG

fractions in vitro. In addition, we performed experiments in Lewis rats immunized with SCW in freund's incomplete adjuvant (FIA) to generate T cell immunity, without spontaneous arthritis. In these rats however, we were not able to elicit a joint inflammation by an intraarticular injection of papain. Such an injection resulted in the release of large amounts of potential cross-reactive cartilage epitopes. These data again indicate that cross-reactivity may not play a key role in the initiation of bacterial arthritis. However, whether cross-reactivity becomes important at later stages in the disease remains to be investigated (Chapter 3).

In addition, we investigated whether lymphocytes of arthritic donor rats are able to specifically migrate to the synovium and subsequently initiate arthritis. Therefore, we performed adoptive transfer experiments with fluorescent T cells obtained from rats with AA, and determined the distibution of these transferred cells in various types of tissue (Chapter 4). If cross-reactivity between bacterial epitopes and cartilage may be involved in the initiation of bacterial-induced arthritis, then one would expect that these bacterial-specific T cells would migrate to the synovium, proliferate in response to cartilage proteoglycans, and initiate arthritis. Interestingly however, we were not able to detect any labelled cells in the joint, suggesting that the cellular infiltrate at the lesion site is mainly host derived. To support these findings further, we performed adoptive transfer experiments to nude Lewis rats, which are reported to lack almost all mature T lymphocytes. These recipients only developed a mild and non-progressive arthritis, which supports the conclusion that a host-derived T-cell population is essential for the development of arthritis by adoptive transfer. Furthermore, these data further indicate that cross-reactivity is not a likely mechanism for the initiation of bacterial arthritis.

Besides the pathogenesis of bacterial-induced arthritis, it was still not completely understood why the Lewis strain is susceptibile and the histocompatible F344 (Fisher) resistant. In this respect, we have earlier described that the F344 strain shows T cell unresponsiveness to SCW and explained the underlying mechanism as immunological tolerance. On the other hand, the susceptibility of the Lewis strain was explained by others as a defect in the hypothalamic-pituitary-adrenal (HPA) axis, leading to an impaired corticosterone responsiveness. We further examined the potential mechanisms which may explain the resistance of F344 rat. It was found that the F344 strain can become susceptible for AA, using a more powerful adjuvant. This indicates that the F344 rat does not lack T effector cells and that neither clonal deletion nor clonal anergy can explain its resistance. Next it was tested, whether the HPA activity was the responsible mechanism for suppression. However, we found identical levels of corticosterone after IL-1α injection in both the resistant F344 and susceptible GF F344. This suggests that corticosterone is not the most important mechanism to suppress disease development in these animals. Finally, we investigated whether active suppression may be responsible for regulating the resistance. This was performed by pretreating the animals with the immunomodulatory drugs cyclophosphamide and cyclosporin A. Our treatment protocol did not make the F344 rat susceptible, indicating that active suppression does not play a role in the induction phase of arthritis. Probably the F344 rat needs to upregulate suppression/tolerance upon bacterial challenge (Chapter 5).

In chapter 6 we further investigated the importance of both the neuroendocrine (HPA axis) and immune system in determining the susceptibility. We observed that the stress-susceptible Wistar rat line (APO-SUS) was completely resistant for AA, while the stress-resistant Wistar rat line (APO-UNSUS) appeared to be moderately susceptible. This indicates that different types of behavioural and neuroendocrinological responses to stress

are correlated to different susceptibility to AA. Furthermore, the germ-free (GF) Wistars were highly susceptible to AA, indicating that the bacterial flora is of prime importance to prevent arthritis. Next it was examined, whether the observed difference in susceptibility was determined by the HPA-axis activity. Therefore, corticosterone levels were analysed in response to IL-1α in APO-SUS and APO-UNSUS Wistar lines and in GF and conventional (CV) rat strains. The data show that the HPA axis activity did not determine the susceptibility to AA. In addition, we measured plasma corticosterone levels around the onset of AA in both GF and CV F344 rats. Whereas no rise was seen in the arthritis-resistant CV rats, a significant increase was observed in the arthritis susceptible GF rats. This suggests that the HPA axis activity is a determinant in modulation of disease activity, rather than being a mechanism to prevent disease.

Finally, in chapter 7 we examined the role of the ß2 integrins in the recruitment of phagocytic cells in joint inflammation. These adhesion molecules appear to be involved in the adhesion and transendothelial migration of phagocytes to the inflammatory site. The ß2 subfamily of integrins comprises 3 members of adhesion molecules: CD11a/CD18, CD11b/CD18 and CD11c/CD18, which are expressed on monocytes and granulocytes. In addition, CD11a/CD18 is also expressed on B and T lymphocytes, although at a low level. We found that selective blocking of the phagocytic cell influx with antibodies directed against the ß2 subfamily was not effective in suppressing AA. Moreover, we examined the same antibodies in an acute non T cell mediated inflammation at different sites in the rat. For this purpose zymosan was injected in either the joint, peritoneum or derm. We observed that pretreatment with αCD11a or αCD11b could only inhibit the influx of phagocytes to an inflamed peritoneum, but not towards an inflamed joint or dermal inflammation. In contrast, treatment with αCD18 was able to block effectively, independent of the site of inflammation. These data indicate that the relative contribution of the individual ß2 integrins seems site and stimulus specific. Interestingly, a recent report showed prevention of the onset of AA with a single injection of antibodies directed against VLA-4 (ß1 integrin). This suggests, that therapy aimed at other adhesion molecules like VLA-4, may give promising results in discrete blocking of the leukocyte influx in particular joint inflammations.

In conclusion, T cells play a pivotal role in the bacterial-induced arthritis models discussed above and T cell tolerance generated and maintained by the bacterial flora is an important mechanism to regulate the susceptibility. However, whether in vivo crossreactivity plays a role in the pathogenesis of these models remains to be further investigated.

Finally, an always returning question is of course the relevance of these bacterial-induced arthritis models. In the last few years several studies have investigated the effects of antibodies directed against the cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) in various arthritis models. For instance, we found that treatment with anti-IL-1 in an established collagen induced arthritis resulted in an amelioration of both inflammation and cartilage destruction, while less effect was observed in the anti-TNF treated animals. On the other hand, when we examined the effects of the same antibodies in an unilateral arthritis, induced by bacterial fragments, we observed exactly the opposite effect. This time anti-TNF treatment was able to signifcantly suppress the level of inflammation, while anti-IL-1 appeared to have a minor effect. In addition to this, studies have been initiated to examine the effects of anti-TNF treatment in rheumatoid arthritis and the first data show significant clinical and laboratory improvements. The findings

indicate that TNF is not only an important regulator in an unilateral arthritis model induced by bacterial fragments, but also in RA. This suggests some similarity in the pathogenesis. Therefore, it not only remains relevant to perform research in various arthritis models, but also points towards a role of bacteria in the pathogenesis of rheumatoid arthritis.

SAMENVATTING

Reumatoïde arthritis (RA) is een chronische vorm van gewrichtsontsteking die bij ongeveer 2% van de volwassen bevolking voorkomt. Het is een systemische ziekte, wat inhoudt dat naast de gewrichten ook andere weefsels zoals bijv. bloedvaten, hart en longen aangedaan kunnen zijn. Tot nu toe is de oorzaak van RA onbekend, maar er zijn aanwijzingen, dat bacteriën een rol spelen bij de inductie van arthritis. Zo is er een relatie gevonden tussen bacteriële infecties en arthritis. Bekende humane voorbeelden hiervan zijn de reactieve arthritis en de verhoogde associatie van arthritis met de ziekte van Crohn en jejunum bypass operaties. Daarnaast is het ook mogelijk om in ratten arthritis op te wekken d.m.v. bacteriën. De twee bekendste voorbeelden hiervan zijn adjuvant arthritis, wat geïnduceerd kan worden door het subcutaan inspuiten van kleine hoeveelheden Mycobacterium in olie, en het streptococcen celwanden-geïnduceerde arthritis model, waarbij grote hoeveelheden celwand-fragmenten van Streptococcus Pyogenes intraperitoneaal worden ingespoten. Met behulp van deze experimentele diermodellen kan men inzicht verkrijgen in de ontstaanswijze van chronische gewrichtsontstekingen en nieuwe therapieën uittesten.

Zowel bij adjuvant arthritis als bij streptococcen celwanden-geïnduceerde arthritis zijn mononucleaire phagocyten (monocyten en macrofagen) en T cellen van essentieel belang. Mogelijke mechanismen die deze chronische arthritis modellen kunnen verklaren berusten enerzijds op een specifieke T cel reactie tegen bacterieel materiaal, wat zich in de gewrichten bevindt, anderzijds op inductie van kruisreactiviteit tegen proteoglycanen van het gewrichtskraakbeen. Immers, bacteriën en proteoglycanen vertonen structureel enige overeenkomsten.

Betreffende de gevoeligheid voor deze vorm van arthritis, is niet alleen de genetische achtergrond bepalend, maar schijnt er ook een regulerende rol weggelegd te zijn voor de bacteriële flora. Zo is de Lewis rat gevoelig, de vrijwel genetisch identieke Fisher ongevoelig en de kiemvrije Fisher weer wel gevoelig voor bacteriële arthritis. Daarnaast suggereren een aantal andere studies, dat ook het neuroendocriene systeem een regulerende rol heeft in de gevoeligheid. Daarbij moet men niet uit het oog verliezen, dat het neuroendocriene en het immuun systeem goed met elkaar kunnen communiceren door middel van overlappende regulatoire mediatoren.

In de eerste studie (hoofdstuk 2) bewijzen we de cruciale rol van T cellen in streptococcen arthritis. Eliminatie van de T cellen m.b.v. antilichamen gericht tegen de CD4 moleculen ten tijde van arthritis inductie, resulteerde in een langdurige resistentie. Zelfs als in een later stadium deze T cellen weer op een normaal niveau waren gekomen, bleken deze proefdieren nog steeds resistent tegen de inductie van bacteriële arthritis.

Vervolgens probeerden we meer inzicht te verkrijgen in het ontstaan van deze vorm van gewrichtsonsteking. Een mogelijke hypothese is kruisreactiviteit tussen bacteriële epitopen en kraakbeen. Zo zijn er een aantal in vitro studies, die dit soort kruisreagerende T cel responsen aantonen. Echter, in vivo bevindingen, die de relevantie van kruisreactiviteit onderbouwen zijn zeer beperkt en deze hypothese is nader getoetst. Zo konden wij in hoofstuk 3 inderdaad in vitro kruisreacties van T cellen aantonen, maar bij analyse van de ontstoken gewrichten bleek het kraakbeen slechts minimaal beschadigd te zijn. Deze bevindingen duiden erop dat kruisreactiviteit waarschijnlijk minder belangrijk is in het ontstaan van bacteriële arthritis. Deze conclusie werd vervolgens indirect onderbouwd door de proeven, waarbij de ziekte passief werd overgebracht d.m.v.

T cellen (hoofdstuk 4). Immers, wanneer wij arthritogene T cellen merken met een fluorescerend label en inspuiten in gezonde ontvanger dieren, konden wij geen accumulatie van deze fluorescerende T cellen in de gewrichten aantonen. Dit zou je niet verwachten, wanneer kruisreactiviteit een rol speelt bij arthritis inductie. Tevens suggereert het, dat de ontstekingscellen (T cellen) in het gewricht voornamelijk afkomstig zijn van de ontvanger zelf. Deze conclusie wordt gesteund door proeven, waarbij de ziekte passief werd overgebracht naar naakte ontvanger dieren. Deze ratten missen functionele T cellen in hun immuun systeem en bleken slechts matig gevoelig te zijn voor deze vorm van arthritis inductie. Alhoewel de combinatie van deze bevindingen het belang van kruisreactiviteit in dit model in twijfel trekt, kunnen we niet uitsluiten dat kruisreactiviteit een rol gaat spelen in latere stadia van ontsteking.

Los van het feit, dat het mechanisme leidend tot gewrichtsontsteking in adjuvant arthritis nog onbekend is, is het ook nog steeds niet duidelijk, waarom de Lewis rat gevoelig is voor bacteriële arthritis, de genetisch identieke Fisher rat ongevoelig en de kiemvrije Fisher rat weer wel gevoelig. Om hier meer duidelijkheid in te krijgen, werden in hoofdstuk 5 een aantal potentiële mechanismen onderzocht, die de ongevoeligheid van de Fisher rat zouden kunnen verklaren. Zo vonden we in deze studie, dat de Fisher ratten geen T effector cellen missen, want ze konden wel een arthritis krijgen, wanneer we een krachtiger adjuvant gebruikten. Vervolgens toonden we aan, dat na injectie van het ontstekingsbevorderend eiwit interleukine-1 (IL-1) er geen verschil was in de endogene produktie van corticosteron in de ongevoelige Fisher en de gevoelige kiemvrije Fisher. Deze bevindingen suggereren, dat niet alleen een hoge activiteit van de hypothalamus-hypophyse-bijnier (HHB) as (het neuroendocrine system) verantwoordelijk kan zijn voor de ongevoeligheid van de Fisher rat. Tenslotte vonden we, dat de F344 rat ongevoelig bleef voor arthritis na voorbehandeling met immunosuppressieve middelen, wat erop duidt dat actieve suppressie geen rol speelt in de inductie fase van de arthritis. Een voorlopige conclusie is, dat de Fisher rat zijn suppressie/tolerantie mechanisme pas activeert bij hernieuwd contact met bacteriën.

In hoofdstuk 6 onderzochten we niet alleen het belang van de bacteriële flora, maar ook of een verschillend type gedrag op stressvolle omstandigheden de gevoeligheid voor bacteriële arthritis kan bepalen. Zo vonden we, dat de stress-gevoelige Wistar rattelijn (APO-SUS) totaal ongevoelig was voor adjuvant arthritis, terwijl de stress-ongevoelige Wistar rattelijn (APO-UNSUS) matig gevoelig bleek te zijn. Dit betekent, dat verschillend gedrag en neuroendocrinologische responsen op stress correleren met een verschillende gevoeligheid voor arthritis. Daarnaast vonden we in deze studie, dat de kiemvrije Wistar ratten zeer gevoelig bleken te zijn voor arthritis. Deze bevindingen duiden erop, dat de bacteriële flora uitermate belangrijk is voor het voorkómen van arthritis. Tenslotte bestudeerden we, of deze verschillen in gevoeligheid verklaard konden worden door een verschil in de aktiviteit van de HHB-as. Echter de resultaten laten zien dat de aktiviteit van de HHB-as meer een regulerende rol vervuld in de ernst van de ontsteking, dan een mechanisme is om de arthritis te voorkomen.

UIteindelijk onderzochten in hoofdstuk 7 de rol van de ß2 integrinen in het rekruteren van ontstekingscellen tijdens arthritis. Immers, wanneer ontstekingscellen naar een ontstekingsgebied migreren, maken deze cellen gebruik van adhesiemolekulen om via de bloedvaatwand de bloedbaan te kunnen verlaten. De ß2 subfamilie van integrinen bestaat uit 3 verschillende type adhesie moleculen: CD11a/CD18, CD11b/CD18 en CD11c/CD18, welke zich bevinden op zowel monocyten als macrofagen. Bovendien bevindt CD11a/CD18 zich ook nog op B en T cellen, alhoewel op een laag niveau. Deze

studie laat zien, dat het blokkeren van de influx van phagocyten m.b.v. antilichamen gericht tegen de ß2 subfamilie (αCD11a, αCD11b en αCD18) geen effect had op het onderdrukken van adjuvant arthritis. Daarnaast onderzochten we ook de effecten van dezelfde antilichamen in een acute niet T cel gemedieerde ontsteking op verschillende plaatsen in de rat. Deze experimenten laten zien, dat voorbehandeling met αCD11a of αCD11b alleen de influx van phagocyten naar een ontstoken buikholte konden blokkeren, terwijl we geen effect zagen in een ontstoken gewricht of huidontsteking. Daarentegen, bleek αCD18 voorbehandeling zeer effectief te zijn, onafhankelijk van de plaats van de ontsteking. Dit suggereert, dat de relatieve bijdrage van de individuele ß2 integrinen plaats en stimulus afhankelijk is.

Concluderend, T cellen spelen een onmisbare rol in bacterieel geïnduceerde arthritis modellen. Daarnaast is T cel tolerantie, opgebouwd en onderhouden door de bacteriële flora, een uitermate belangrijk mechanisme om de gevoeligheid voor arthritis te bepalen en schijnt neuroendocrine regulatie minder essentieel. Tenslotte, lijkt het onwaarschijnlijk, dat kruisreactiviteit met kraakbeen een belangrijke rol speelt bij het ontstaan van bacteriële arthritis.

DANKWOORD

De totstandkoming van dit proefschrift werd mede mogelijk door de steun van vele anderen. Alhoewel ik me realiseer, dat je gemakkelijk iemand vergeet, wil ik toch bij deze een poging wagen om al diegene te bedanken, die op enigerlei wijze een bijdrage hebben geleverd aan dit proefschrift.

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Lieve Daniëlle, jij was echt onmisbaar in elk opzicht. Een dubbel-promotie is toch wel heel bijzonder!

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 7 augustus 1961 te Oisterwijk. In 1981 behaalde hij het diploma Gymnasium ß aan het Stedelijk Gymnasium te 's-Hertogenbosch. In dat zelfde jaar werd een begin gemaakt met de studie biologie aan de Rijks Universiteit Utrecht. In januari 1985 behaalde hij zijn kandidaatsexamen Biologie. Daarna volgde een bijvak Moleculaire Genetica aan de Rijks Universiteit Utrecht (Prof.dr. van Arkel), een hoofdvak Medische Microbiologie op de afdeling Immunology and Medical Microbiology (Prof. W.W. Hauswirth) aan de University of Florida, in Gainesville, USA en een hoofdvak Veterinaire Immunologie op de afdeling Pathologie van het Rijksinstituut voor Volksgezondheid en Milieuhygiene (Dr. H. van Loveren). Het doctoraal examen B5* (Medische Biologie) werd behaald in november 1987. In 1988 werd een postdoctoraal onderzoek uitgevoerd bij de vakgroep Immunology and Medical Microbiology aan de University of Florida op uitnodiging van Prof. Hauswirth. Van februari 1989 tot mei 1994 was hij werkzaam als onderzoeker op het laboratorium reumatische ziekten van het Academisch Ziekenhuis St. Radboud te Nijmegen. Vanaf mei 1994 is hij werkzaam op de medische afdeling van de firma Roussel als clinical research associate.

STELLINGEN

behorende bij het proefschrift
"Regulation of bacterial induced arthritis".

1. T-cel tolerantie, opgebouwd en onderhouden door de bacteriële flora, is een uitermate belangrijk mechanisme om de gevoeligheid voor arthritis te bepalen.
   Dit proefschrift

2. Bij het ontstaan van bacteriële arthritis lijkt het onwaarschijnlijk dat kruisreactiviteit met kraakbeen een belangrijke rol speelt.
   Dit proefschrift

3. Bij de keuze van radionucliden voor radioimmunotherapie zal de veiligheid van het betrokken medisch personeel een steeds belangrijkere rol gaan spelen.
   Martijn Gerretsen, Proefschrift 1993, Vrije Universiteit Amsterdam

4. Lever macrofragen kunnen proliferen.
   Rein Hoedemakers, Proefschrift 1993, Universiteit Groningen

5. Het feit dat de SCW-arthritis gebruik maakt van dode bacteriën is een prettige bijkomstigheid, nu blijkt dat *Streptococcus pyogenes* ook vleesetende capaciteiten blijkt te bezitten.

6. De anti-rookcampagnes houden te weinig rekening met het feit, dat nicotine ook heilzame effecten kan uitoefenen, zoals het vertragen of voorkómen van ziekten als Parkinson en Alzheimer.

7. Het is de vraag wie het meeste voordeel heeft van een promotie-onderzoek: de promotor of de promovendus.

8. Het gebruik van afkortingen binnen de pharmaceutische industrie neemt zulke vormen aan, dat deze wellicht binnenkort in boekvorm kunnen worden uitgegeven.

9. Het is goed dat Nederland niet Italië is, anders zou Joop van den Ende nu premier zijn.

10. Het werken met naakte ratten is een schamele troost voor kalende wetenschappers.

Nijmegen, 9 november 1994 — Lex van de Langerijt

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