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The Endogenous Adjuvant Squalene Can Induce a Chronic T-Cell-Mediated Arthritis in Rats

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Squalene is a cholesterol precursor, which stimulates the immune system nonspecifically. We demonstrate that one intradermal injection of this adjuvant lipid can induce joint-specific inflammation in arthritis-prone DA rats. Histopathological and immunohistochemical analyses revealed erosion of bone and cartilage, and that development of polyarthritis coincided with infiltration of $\alpha\beta^+$ T cells. Depletion of these cells with anti-$\alpha\beta$ TcR monoclonal antibody (R73) resulted in complete recovery, whereas anti-CD8 and anti-$\gamma\delta$ TcR injections were ineffective. The apparent dependence on $CD4^+$ T cells suggested a role for genes within the major histocompatibility complex (MHC), and this was concluded from comparative studies of MHC congenic rat strains, in which DA.1H rats were less susceptible than DA rats. Furthermore, LEW.1AV1 and PVG.1AV1 rats with MHC identical to DA rats were arthritis-resistant, demonstrating that non-MHC genes also determine susceptibility. Some of these genetic influences could be linked to previously described arthritis susceptibility loci in an F2 intercross between DA and LEW.1AV1 rats (ie, *Cia3*, *Oia2* and *Cia5*). Interestingly, some F2 hybrid rats developed chronic arthritis, a phenotype not apparent in the parental inbred strains. Our demonstration that an autoadjuvant can trigger chronic, immune-mediated joint-specific inflammation may give clues to the pathogenesis of rheumatoid arthritis, and it raises new questions concerning the role of endogenous molecules with adjuvant properties in chronic inflammatory diseases. *(Am J Pathol 2000, 156:2057–2065)*

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that leads to impaired joint function, severe pain, and reduced life expectancy.[1] This putative autoimmune disease affects more than 2.5 million people in the United States[2] and thus constitutes a serious health and cost problem for individuals and societies. A major obstacle to the development of rational treatment strategies is that the disease mechanisms and the causative environmental and genetic factors remain largely unknown. Clues may come from experimental arthritis; one intriguing observation in this respect is that joint inflammation can be induced in arthritis-prone rat strains by a variety of exogenous molecules that trigger the immune system nonspecifically, ie, adjuvants. This has been demonstrated not only with microbial cell wall structures, such as muramyldipeptide,[3] lipopolysaccharide, trehalosedimycolate, and β-glucan,[4] but also with oils such as pristane.[5] Recently, it was suggested that this mode of arthritis induction may also apply to endogenous molecules, because in a pilot experiment, one intradermal injection of the cholesterol-precursor squalene ($C_{30}H_{50}$) precipitated joint inflammation in inbred DA rats.[4] The possibility that self-molecules other than proteins and peptides can trigger arthritis could have wide implications for putative autoimmune diseases such as RA, especially if the joint inflammation exhibits features associated with such diseases. Consequently, we here characterize squalene-induced arthritis (SIA) with an emphasis on determining the similarities with RA. This study delineates the disease course, the influence of sex, the organ- and tissue-specificity of inflammation, and the dynamics of the joint inflammation including infiltrating cell types, the influence of T cells, the humoral and cellular reactivity to the arthritogenic cartilage autoantigens rat collagen type II (CII) and cartilage oligomeric protein (COMP),[6,7] the influence of DA major histocompatibility complex (MHC) and non-MHC genes on disease susceptibility, and the possibility of linkage of non-MHC gene effects to previously described disease susceptibility loci.

Materials and Methods

Rats

Inbred DA, DA.1H, LEW.1AV1, LEW.1F, and PVG.1AV1 rats were originally derived from Zentralinstitut für Ver-

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suchstierzucht (Hannover, Germany). The genetics and characteristics of the rat strains used are described elsewhere.[8] The inbred rats and F2 (DA×LEW.1AV1) intercrosses were bred, kept, and used under specific-pathogen-free conditions at the Biomedical Center in Uppsala or the Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden. The rats were sex- and age-matched for each experiment. They were kept in a climate-controlled environment with 12-hour light/dark cycles, housed in polystyrene cages containing wood shavings, and given free access to standard rodent food and water. Experimental procedures involving animals were performed according to guidelines provided by the central board for animal experiments at the Swedish Department of Agriculture and were approved by the Ethical Board for animal experiments in Stockholm-North.

Induction and Evaluation of Squalene-Induced Arthritis (SIA)

Arthritis was induced under anesthesia by an intradermal injection at the base of the tail with 200 or 300 μl squalene ($C_{30}H_{50}$, density = 0.86 g/ml, derived from shark liver), more than 99.8% pure according to the supplier (Sigma Chemicals, St. Louis, MO). Saybolt (Gothenburg, Sweden) analyzed the squalene for nitrogen content, using an assay for detection of protein residues in oils (ASTM D4629). No nitrogen could be detected with the detection limit of 0.0001% w/w, which equals a protein content of <0.0006% w/w, assuming 16% nitrogen in the protein. Thus, in squalene-induced arthritis each rat receives <1 μg of shark liver protein, a dose that is extremely unlikely to be responsible for arthritis induction. For comparison, 150–500 μg/rat of cartilage collagen type II is commonly used to induce collagen-induced arthritis. Arthritis development was monitored every second to fourth day by a macroscopic scoring system ranging from 0 to 4 for each of the four limbs (1, enlargement of one type of joint; 2, enlargement of two types of joints; 3, more than two types of joints involved; 4, severe arthritis in the whole paw), yielding a score of 0 to 16 per animal. From day 40 postinduction (p.i.), and every tenth day thereafter, the appearance of arthritis in each individual rat was also graphically depicted on paw maps.

T Cell Depletion

Monoclonal antibodies (mAbs) purified from hybridoma supernatants using standard protein G affinity chromatography were solved in phosphate-buffered saline (PBS) and injected i.p. in arthritic DA rats at a dose of 1 mg/rat.

The mAb used were directed against $\alpha\beta$-TcR (R73, complete depletion at 0.08 mg/rat),[9,10] $\gamma\delta$-TcR (V65, 93–96% depletion at 0.1–0.5 mg/rat)[11,12] CD8 (ox-8, complete depletion at 0.1 mg/rat),[13] and TNP (D10, used as negative control). The hybridomas were kindly provided by Dr. Tomas Hünig, Würzburg, Germany (V65 and R73), Dr. Birgitta Heymann, Uppsala, Sweden (D10), and the late Dr. Alan Williams, Oxford, UK (ox-8).

T Cell Proliferation Assay

Inguinal lymph node cells from arthritic and normal DA rats were suspended at 1×10^6 cells/ml in DMEM supplemented with 5% FCS, penicillin (100 U/ml), glutamine (2 nmol/L) and streptomycin (100 μg/ml), all from Sigma. The cells were plated in 96-well flat-bottom cell culture plates (Nunc, Roskilde, Denmark), 0.2 ml per well. Antigens (Ag) were added to triplicates of wells, dissolved in 10 μl PBS, pH 7.4, to the final concentrations: 10 μg/ml of bovine COMP or rat CII, 3 μg/ml of Con A. As control, 10 μl PBS were added to the wells. The cells were incubated for 72 hours at 37°C in 5% CO_2, and proliferating cells were labeled with 1 μCi of ^{3}H-thymidine per well for the final 18 hours before cell harvest. Incorporation of label was determined by liquid scintillation counting, using a Beckman scintillator.

Dissection of Organs and Decalcification of Paws

Left hind paws were, after skin removal, put in a decalcifying solution, which was changed daily until the decalcification of bone was complete (0.3 mol/L EDTA, 4 mmol/L PVP, 0.1 mol/L Tris-HCl, pH 6.95).[14] Right hind paws, inguinal lymph nodes, spleen, spinal cord, lung, liver, kidney, and skin were dissected out, snap-frozen in isopentane, and stored at −80° until cryosectioning and immunohistochemical staining.

Immunohistopathological Analyses

Hematoxylin-Eosin Staining

Paraffin-embedded sections (8 μm) of formalin-fixed decalcified rat paws were dewaxed, rehydrated, and stained with HTX (Mayer's hematoxylin, Apoteksbolaget, Sweden) and eosin (Sigma) according to standard protocol, and mounted with Mountex (Histolab, Gothenburg, Sweden).

Immunohistochemical Staining for Cell Surface Markers

Cryostat sections (8 μm) of decalcified rat paws were mounted on gelatin coated microscope slides (Novakemi, Stockholm, Sweden), dried, and stored at −80°C. The sections were fixed in acetone (KeboLab, Spånga, Sweden). Endogenous peroxidase was blocked with 1% H_2O_2 and 2% NaN_3 in PBS in a coplin jar, followed by three washes in PBS, and preincubation with 2% normal horse serum (Vector Laboratories Inc., Burlingame, CA). All incubations were at room temperature in a humid chamber unless otherwise indicated. The slides were incubated overnight with a panel of mouse mAbs against the following cell surface markers: $\alpha\beta$-TcR (R73), $\gamma\delta$-TcR (V65), CD8 (ox-8), CD4 (W3/25), CD11b/c (ox-42, purchased from Serotec, Novakemi), MHC class II (ox-6), and isotype-matched control (IgG_1, Dakopatts, Denmark), respectively, at a concentration of 0.3–10 μg/ml in

2% normal rat serum, 1% BSA, 0.02% sodium azide diluted in PBS. The W3/25 and ox-6 hybridomas were kindly provided by late Dr. Alan Williams (Oxford, UK). After washing 3× in PBS, 6 μg/ml of biotinylated secondary antibody, absorbed against rat Ig (biotin horse-anti-mouse IgG, Vector Labs), in 2% normal rat serum in PBS was added. After washing 3× in PBS, slides were incubated with avidin-biotin horseradish peroxidase (Vectastain ABC Standard, Kemila, Sweden) and washed in 3× PBS. Color reaction was developed in coplin jars in 3-amino-9-ethyl-carbazole (AEC, Sigma; 9 ml of 2.5 mg/ml AEC in dimethylsulfoxide, Merck, Germany), 75 ml 0.02 mol/L sodium acetate, pH 5.5, and 6 μl 30% H_2O_2), washed, counterstained with HTX, and mounted.

Immunohistochemical Staining for Fibrin Deposition

Detection of fibrin in paws was performed as for the surface markers. The anti-fibrinogen monoclonal goat-anti-rat antibody (Sigma) and the isotype-matched control were used at a concentration of 1 μg/ml. As secondary antibody, 3 μg/ml of biotinylated donkey-anti-goat antibody (Jackson Immunoresearch Laboratories, West Grove, PA) were used. The specificity of the primary antibody was verified by pre-absorption of this antibody by rat fibrinogen (Sigma; 20 μg rat fibrinogen/μg antibody).

Immunohistochemical Staining for Proliferating Cell Nuclear Antigen (PCNA)

Detection of PCNA in paws was performed as for the surface markers, with minor alterations. Cells were permanently perforated using 0.03% Triton (Merck, Darmstadt, Germany) in PBS as a diluent. A monoclonal antibody against PCNA (isotype IgG_{2a}, clone PC10) was used at a concentration of 4 μg/ml (Dakopatts). Omission of primary mAb was used as negative control. ABC Elite (Vectastain, Immunkemi, Järfälla, Sweden) was used as amplifier and diaminobenzene (DAB, Vector Labs) augmented with Ni was used as color developer. The sections were not counterstained.

Genetic Analysis

The tip of the tails from the F2 intercrosses were collected, and genomic DNA was purified according to a standard protocol.[15] Genotyping was performed by PCR amplification of variable number tandem repeats (microsatellites) that were polymorphic between the two parental strains, essentially as previously described,[16] except ^{33}P-γATP was used to label one of the primers in each pair. The following genomic markers within arthritis-linked chromosome intervals were used: D4Mit12, D4Arb24, D4Mgh3 (markers for *Cia3* = *OiaW*), D4Mgh7, D4Wox14, EN4, D4Mit27 (markers for *Oia2*) and IGFBP4, D11Mit58 (mouse marker), D10Mgh1, D10Rat2 (markers for *Cia5* = *Oia3*).[17,18] The genotypes for each marker and animal were determined and denoted D for DA-specific alleles and L for LEW.1AV1 alleles, yielding the genotypes DD, DL, or LL. For each marker, the F2 hybrids were categorized according to genotype, after which the three groups were compared for arthritis phenotypes. The probability that phenotype differences between the groups occurred by chance was calculated using the Kruskall-Wallis test.

Quantification of Humoral Anti-CII or Anti-COMP Immunity

Plasma was collected *postmortem*, left on ice until centrifugation, and stored at −80° until analysis. IgG and IgM antibody titers to rat CII or bovine COMP were determined using a standard ELISA.[19] In brief, Maxisorp MicroELISA plates (Nunc, Roskilde, Denmark) were coated with 10 μg/ml of CII or COMP in PBS. Bound antibody was detected with biotinylated F(ab′)2 fragment goat anti-rat IgG or IgM Fc fragment-specific (Jackson) and alkaline phosphatase-conjugated streptavidin (Jackson) and quantification of bound enzyme was performed with a p-nitrophenyl containing substrate buffer in an E-max spectrophotometer (Molecular Devices, Sunnyvale, CA). Each serum sample was measured in duplicate.

Quantification of Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) in Serum, Plasma, and Lymph Node Cell Culture Supernatants

Plasma and serum were prepared from DA, LEW.1AV1, and PVG.1AV1 rats sacrificed 20 days after squalene-injection, and from naive animals from the same strains matched for age and sex. In addition, inguinal lymph nodes were dissected out from each animal, and single cell preparations were cultured in DMEM supplemented with 10% FCS, penicillin (100 U/ml), glutamine (2 nmol/L), and streptomycin (100 μg/ml), all from Sigma, at a concentration of 2×10^6 cells/ml and incubated for 24 hours in 37°C. The supernatants, sera and plasma were stored at −20° until quantification of rat TNF-α and IL-1β using ELISA kits (Nordic BioSite, Stockholm, Sweden). The detection limits of TNF-α and IL-1β were 31 and 25 pg/ml, respectively.

Nephelometric Determination of Fibrinogen in Plasma

The fibrinogen levels in plasma collected from DA, DA.1H, LEW.1AV1, and PVG.1AV1 rats at day 12 after squalene induction were determined using nephelometry, as previously described,[20] and compared with plasma levels from normal, age-matched animals and from arthritic DA rats at days 16 and 20 postinduction.

Statistical Methods

Non-parametric two-tailed ranking tests were used in all statistical analyses (Mann-Whitney and Kruskall-Wallis). P values <0.05 were considered significant.

Results

Macroscopic and Histopathological Appearance of SIA in DA Rats

After squalene injection, macroscopic signs of inflammation were evident only in the joints. Arthritis developed in 100% of DA rats, with no apparent sex-linked difference (Figure 1). The mean day of onset was 13 days postinduction (p.i.), and the first signs of arthritis typically appeared symmetrically in ankles and metatarsal joints of all paws, and progressed to include larger joint areas and finger joints. Macroscopically, no signs of inflammation were detected in knee, elbow, or hip joints. The mean max score was reached at day 21 p.i. A histopathological examination at this time point revealed a hypertrophic synovial tissue with pannus invading the joint space (Figure 2A). Infiltrating neutrophils and other inflammatory cells (exemplified by $\alpha\beta$+ T cells in Figure 2B) were abundant within the joints as well as in the surrounding

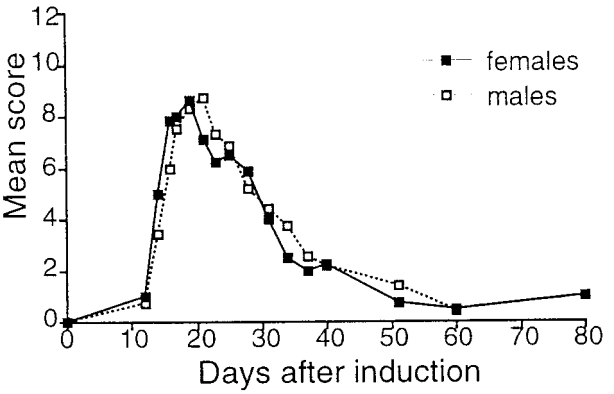

Figure 1. Macroscopic appearance of squalene-induced arthritis (SIA) depicted as mean score in female (filled squares, n = 8) and male (empty squares, n = 9) DA rats. The arthritis is typically monophasic, leaving no permanent deformation of the joints.

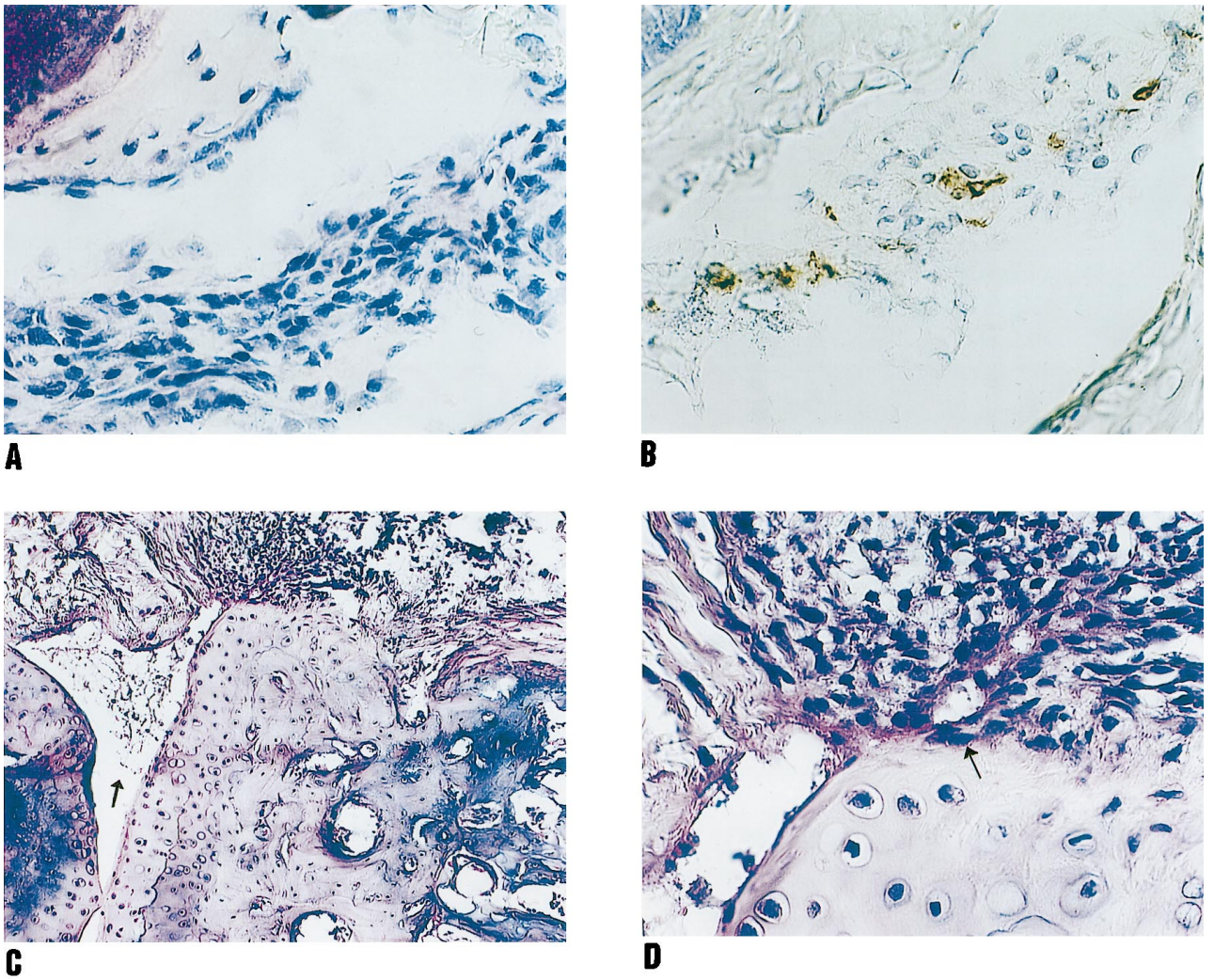

Figure 2. Microscopic appearances of arthritic joints in DA rats, day 20 after squalene induction. **A:** Pannus infiltrating the joint (HTX/Eosin, 210 × magnification) with (**B**) infiltrating $\alpha\beta^+$ T cells (IHC staining, detected using the avidin-biotin complex with diaminobenzene as substrate; original magnification, ×210). **C:** Overview of the joint. **Arrow** denotes typical fibrin deposition in the joint space (HTX/eosin; original magnification, ×55). **D:** The same section; original magnification, ×210. **Arrow** indicates chondrolysis.

Table 1. Immunohistochemical Analysis of Squalene-Induced Arthritis

Paw examined	Days after induction	αβ TcR (R73)	γδ TcR (V65)	CD11b/c (ox-42)	MHC cIII (ox-6)	PCNA (PC10)
Normal*	0	−	−	+	+	(+)
Pre-arthritic*	11	−	−	+	+	(+)
Arthritic, acute*	16	++¶	(+)	+++‡	++++§	+++¶
Arthritic, chronic†	80	++§	+	++++§	++++§	+++§

−, 0–1%; (+), <1%; +, 1–10%; ++, 10–25%; +++, 25–50%; ++++, >50% positively stained cells.
*DA rats.
†F2(DA×LEW.1AV1) rats.
‡$P < 0.05$
§$P < 0.01$
¶$P < 0.001$

tissues. Osteolysis, chondrolysis, and synovitis were recorded in all paws examined (Figure 2, C and D); in some cases, a complete lysis of the bone was noted. Fibrin exudation was observed in affected joint spaces (Figure 2C). After maximum score was reached, the joint inflammation gradually subsided and left few or no signs of macroscopic damage or ankylosis.

Dynamic Histochemistry of Joints and Organs in SIA

That only joints appeared affected led us to determine the dynamics of inflammation in SIA. Three to four joint specimens from DA rat paws at different stages of disease development were therefore stained with inflammation-related cell surface markers. The staining frequencies for each specimen were calculated and ranked by a histopathologist blinded to the experiments. The results in Table 1 demonstrate that staining for CD4, CD8, αβ TcR, CD11b/c, MHC class II, and PCNA (early cell activation)[21] were significantly up-regulated after arthritis onset, as compared to normal joints, whereas this was not the case for paws taken just before arthritis onset (pre-arthritic). The result was the same when staining for fibrinogen; fibrin deposition was recorded on the cartilage facing the joint space, but we were unable to identify quantitative differences between normal and pre-arthritic paws, whereas an extensive fibrin deposition occurred in the inflammatory cell area in arthritic paws. γδ+ T cells were only slightly up-regulated in the inflamed paws. Immunohistochemical stainings performed on spinal cord, skin, liver, lung, salivary gland, kidney, and diaphragm from arthritic animals revealed no inflammatory cell infiltration. Hence, the inflammation was joint-specific and we were unable to detect signs of arthritis before T cells' infiltration into joints.

Effects of T Cell Depletion on Arthritis Development

DA rats with established arthritis were given one i.p. injection of mAb at day 12 p.i. (Figure 3). Injection with anti-γδ TcR, anti-CD8, or isotype-matched irrelevant anti-TNP Ab did not affect disease development. In contrast, anti-αβ TcR mAb (R73) abolished arthritis within 1 or 2 days, for at least 10 days ($P < 0.01$, R73 *versus* control mAb). At approximately day 30 p.i., the arthritis recurred and αβ T cells could be detected in the draining inguinal lymph nodes.

Cellular and Humoral Reactivity toward CII and COMP

The dramatic influence of αβ T cells led us to investigate the proliferative response of these cells to arthritogenic joint-derived autoantigens. Cell proliferation assays of inguinal lymph node cells from four normal DA rats and four arthritic DA rats sacrificed 13 days p.i. revealed no increased proliferation after stimulation with CII or COMP. Stimulation with con A induced proliferation in both groups of animals, although with a tendency for more proliferation in animals previously provoked with squalene ($P = 0.07$, results not shown).

Humoral reactivity toward CII or COMP was measured in all strains and in the F2 (DA×LEW.1AV1) intercrosses. All animals lacked reactivity, except a few F2 intercross

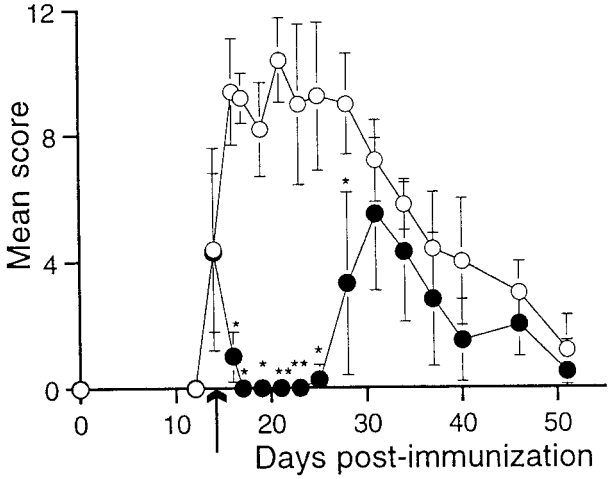

Figure 3. Effect of therapeutic $\alpha\beta^+$ T cell depletion in DA rats with squalene-induced arthritis. Rats injected i.p. with depleting mAbs against αβ TcR (R73, filled circles; $n = 4$) recovers from SIA within 1 day, whereas DA rats given an i.p. injection with nondepleting control mAbs (D10, open circles; $n = 5$) still have a severe arthritis. The **arrow** indicates the time of administration of mAbs (day 14 p.i.). *$P < 0.05$, **$P < 0.01$.

Table 2. Susceptibility to Squalene-Induced Arthritis in Different Rat Strains

Strain	Non-MHC	MHC	Sex/nr	Incidence (%)	Day of onset	Max score*
DA.1H	DA	h	F/9	100	15 ± 1.5§	6.4 ± 2.4‡
			M/8	88†	18 ± 4.8†	4.3 ± 2.5‡
DA	DA	avl	F/8	100	13 ± 1.1	9.1 ± 1.9
			M/9	100	14 ± 1.8	9.2 ± 1.6
LEW.1AV1	LEW	avl	F/8	25‡	21 ± 2.8†	5.5 ± 3.5
			M/5	0	—	—
PVG.1AV1	PVG	avl	F/8	0	—	—
			M/8	0	—	—

All comparisons were made with sex-matched DA rats. nr, number of rats.
*From arthritic rats only.
†$P < 0.05$
‡$P < 0.01$
§$P < 0.001$

animals, which had low levels of IgG and IgM against CII or COMP (data not shown). However, these reactivities did not coincide with arthritis onset, severity, or chronicity.

Detection of the Pro-Inflammatory Cytokines TNF-α and IL-1β

Using ELISA, neither of the pro-inflammatory cytokines, TNF-α and IL-1β, could be detected in sera and plasma from naive and squalene-injected DA, LEW.1AV1, and PVG.1AV1 rats, even though the squalene-injected DA rats were arthritic when the samples were collected (20 days p.i.). Neither did draining lymph node cell culture supernatants from any of the animals contain detectable amounts of cytokines (data not shown).

Influence of Sex, MHC, and Non-MHC Genes

Because αβ T cells are important for arthritis development, the influence of MHC genes was investigated using MHC congenic strains. MHC dependence in SIA was clear because DA.1H had significantly lower incidence, maximum score, and later day of onset compared to DA (Table 2). This observation was supported in another experiment, in which LEW.1F animals were significantly more susceptible than LEW.1AV1 rats (results not shown). Sex differences were also recorded in DA.1H and LEW.1AV1, where fewer males developed SIA (Table 2). Furthermore, non-MHC genes strongly influenced the disease course and severity, since the MHC-identical LEW.1AV1 and PVG.1AV1 strains were predominantly or completely arthritis-resistant, respectively.

Genetic Analysis of Influences on Arthritis Susceptibility and Plasma Fibrinogen Levels of Non-MHC Loci in a F2 (DA×LEW.1AV1) Intercross

A genetic analysis performed on 46 female F2 (DA×LEW.1AV1) progeny revealed that development of SIA was linked to DA alleles at the microsatellite markers for *OiaW*, *Oia2*, and *Oia3* (D4Mit12, D4Wox14, and D10Mgh1 respectively, the reference markers for each chromosome region) as shown in Figure 4.

It was noted that arthritis in several F2 hybrids appeared progressive. All F2 females were therefore monitored until 80 days postinduction, together with sex-matched DA and LEW.1AV1 rats. Interestingly, 21% (6/29) of the diseased F2 progeny developed a chronic disease (Table 3), with new intracarpal/intratarsal or peripheral interphalangeal joints becoming affected and active at days 60 and 80. No chronicity was observed in DA rats. Furthermore, 38% (11/29) of the diseased F2 crosses and 12% (1/8) of the DA rats were ankylotic, in which ankle joints were enlarged and deformed but did not appear inflamed. Immunohistochemical stainings of paws with chronic arthritis revealed a distribution of inflammatory cells similar to that in acute arthritis (Table 1).

Compared to normal DA rats, plasma fibrinogen levels were significantly elevated ($P < 0.001$) in arthritic DA rats, but not in pre-arthritic DA rats (Figure 5). In the predominantly arthritis-resistant LEW.1AV1 rats, there was a tendency for elevated levels of fibrinogen in pre-arthritic rats. Interestingly, increased plasma levels in F2 progeny at day 12 (pre-arthritic) were linked to *Oia2* and *Oia3* (*P* values 0.04 and 0.02, respectively), suggesting that fibrinogen levels can serve as a prognostic marker for arthritis development.

Discussion

The discovery that an endogenous lipid can trigger arthritis in rats could have wide pathogenetic implications in humans. The aim of this study, therefore, was to characterize SIA with special reference to the common chronic inflammatory disease RA.

We demonstrate that similarities between RA and SIA include genetic influences of MHC and sex, ie, female preponderance. Furthermore, it appears that SIA fulfils four out of seven inclusion criteria required for the diagnosis of RA.[22] In short, these similarities are long-lasting symmetric arthritis involving a minimum of three groups of joints, including hand joints, which leads to bone erosion. The remaining three criteria, ie, the presence of morning stiffness, rheumatic nodules, and rheumatoid factors,

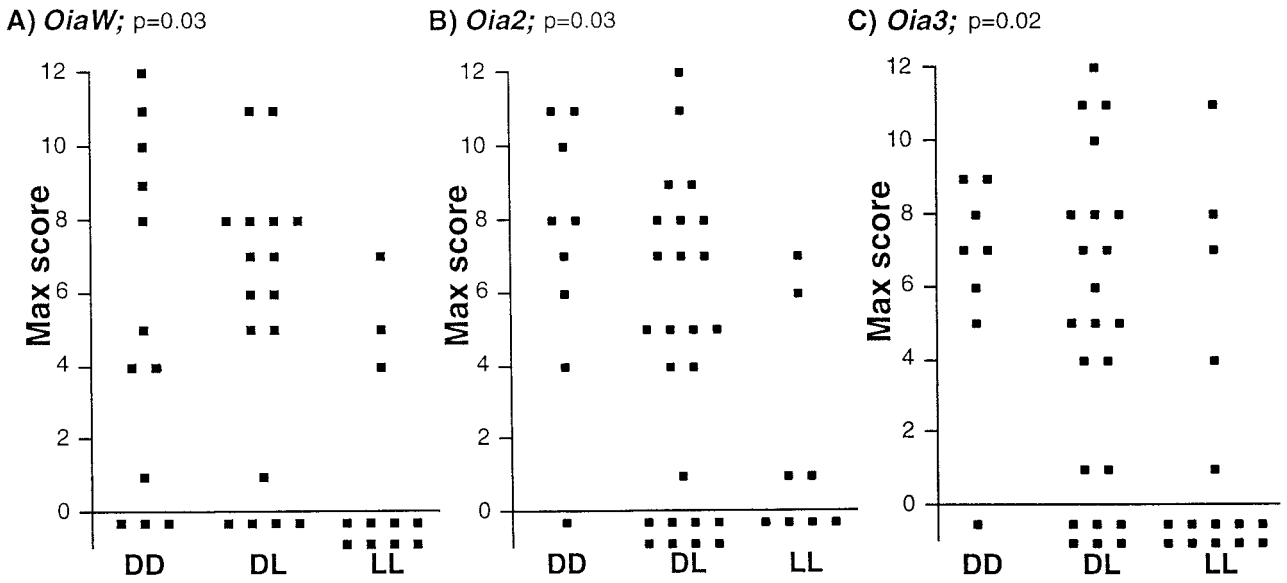

Figure 4. Genetic distribution of SIA-induced F2(DA×LEW.1AV1) intercrosses with regards to the arthritis susceptibility-linked regions: **A**, OiaW; **B**, Oia2; and **C**, Oia3. **Dots** represent the specific genotype of each individual. The *y* axis represents the maximum arthritis score for each individual. The Kruskall-Wallis test was used in the statistical analyses.

were not investigated. Considering the fact that inflammation was restricted to the joints, we examined affected animals for the presence of antibodies against CII, an autoreactivity present in some RA patients.[23–26] We did not detect such antibodies in susceptible DA rats, but an immunological involvement in the pathogenesis was nevertheless clear, since depletion of T cells expressing $\alpha\beta$ TcR completely abrogated the disease. In contrast, depletion of CD8+ T cells and $\gamma\delta$ TcR+ cells did not ameliorate the disease, which points to a pathogenic role for $\alpha\beta$ TcR+ CD4+ T cells. Because arthritis onset correlated with T cell infiltration into joints, we examined the possibility that T cells specific for cartilage antigens were activated by squalene, but there was no increased proliferation of draining lymph node cells from arthritic animals after *in vitro* stimulation with the arthritogens tested, ie, CII and COMP.[6,7]

Besides the possibility that autoreactivity accounts for the restriction of inflammation to the joints, we considered two alternative possibilities. One explanation could be that joint tissues are particularly responsive to adjuvant injections and therefore preferentially attract circulating activated T cells. In this scenario, inflammation markers would be up-regulated before infiltration of lymphocytes. But, when comparing normal joints to joints taken just before expected arthritis onset, we were unable to detect up-regulation of MHC class II, fibrin deposition, or PCNA before T cell infiltration. However, the hypothesis of joint sensitivity cannot be rejected by our results, because we may not have monitored the critical putative proinflammatory marker(s). Therefore, a continued search for early proinflammatory changes of joint phenotypes is of importance. A second possible explanation for joint restriction in adjuvant arthritis may be that the injected adjuvant

Table 3. Susceptibility and Chronicity in Female F2 (DA×LEW.1AV1) Intercrosses Compared with Sex-Matched Parental Animals

Strain	No. of animals	Incidence (%)	Chronic arthritis (% of diseased animals)
DA	8	100	0
LEW.1AV1	8	25	0
F2(DA×LEW.1AV1)	46	63	21

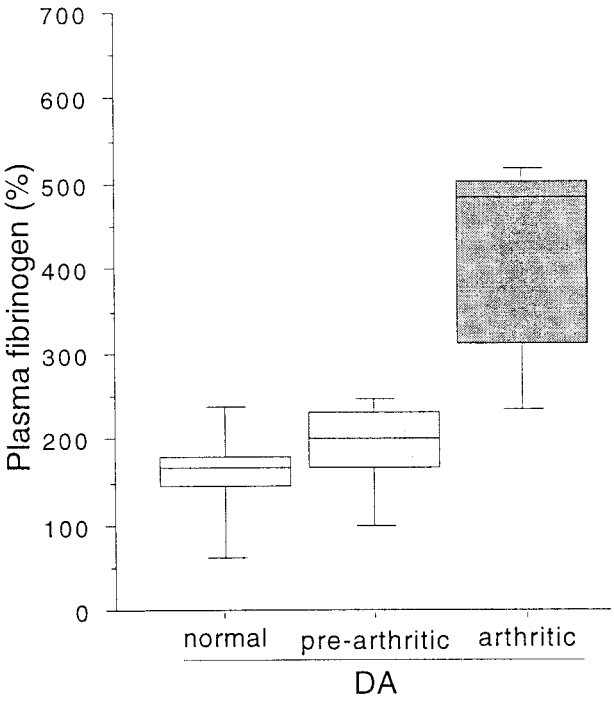

Figure 5. Plasma fibrinogen levels of normal, pre-arthritic, and arthritic DA rats, respectively. Levels are presented as percentage of normal control plasma. The Kruskall-Wallis test was used in the statistical analyses, giving a *P* value of 0.0013.

preferentially accumulates in joint tissues. We are presently addressing this hypothesis by tracking tritium-labeled squalene *in vivo*.

To understand the mechanisms whereby squalene induces arthritis, it will also be important to determine how the lipid affects the immune system. Interestingly, squalene is used commercially for vaccination and immunization purposes[27] to potentiate immune responses. This capacity to nonspecifically stimulate the immune system probably accounts for the arthritis development, considering the recent demonstration that many structurally unrelated adjuvant molecules can cause arthritis.[4] It is possible, however, that squalene is also targeted by adaptive immunity, as it is becoming increasingly clear that nonclassical antigens such as lipids can evoke T cell responses, for example, after presentation on CD1 molecules.[28,29] Interestingly, the squalene precursor isopentenyl pyrophosphate is one molecule that has been shown to be a T cell ligand.[30] Furthermore, it has recently been suggested that anti-squalene antibodies (Abs) can develop as a consequence of vaccination with vaccines containing squalene, and that these Abs are associated with pathology in the Gulf War syndrome (Dr. Pam Asa, personal communication).

Irrespective of how the immune system is activated, the process is probably influenced by the metabolism of squalene. It may therefore be interesting to modulate the metabolism of this arthritogen and to determine how this affects arthritis development in susceptible and resistant strains. Because oxidized lipids are being implicated as playing a role in RA,[31] it would also be interesting to investigate whether the degree of oxidation of squalene influences the disease outcome.

One strategy to unravel the pathogenesis of SIA is to identify the disease-associated genes, and therefore, an initial genetic analysis was performed. First, there was a significant female preponderance in DA.1H rats, and only females were affected in the few cases where SIA developed in LEW.1AV1 and LEW.1F rats. In contrast, there was no gender dimorphism in the DA strain. It is possible that these extraordinarily arthritis-prone rats harbor a collection of homozygous susceptibility alleles that override the effects of sex, possibly sex hormones, on the immune system, as previously suggested in the NZB/W model system for lupus nephritis.[32] Second, an influence of MHC genes was demonstrated by the higher susceptibility of DA rats compared to DA.1H rats, ie, DA rats with a different MHC haplotype ($RT1^h$ instead of $RT1^{av1}$). We are presently establishing intra MHC recombinant strains to identify the important gene regions, which could be class II genes, but also class I genes or the class III genes for TNF-α, heat shock proteins, and complement. Thirdly, the relative resistance of LEW.1AV1 rats and complete resistance of PVG.1AV1 rats demonstrates that non-MHC genes also determine susceptibility, since these strains express the MHC haplotype of the susceptible DA rats ($RT1^{av1}$). Interestingly, LEW.1AV1 rats have previously been reported to be highly arthritis susceptible,[33] and have been used to identify new arthritogenic antigens and adjuvants.[4,7] Therefore, the resistance of LEW.1AV1 rats recorded here suggests that squalene is a weakly arthritis-inducing substance. In fact, the susceptibility profile of SIA in MHC congenic strains is similar to that recorded in arthritis induced with IFA,[34] ie, oil-induced arthritis (OIA).[35] It was therefore reasonable to suspect that susceptibility to SIA would be linked to the non-MHC loci recently reported in OIA, based on a whole genome scan in a F2 (DA $\times$ LEW.1AV1) intercross.[18] We confirmed this supposition by linking SIA to *OiaW*, *Oia2* and *Oia3* in the same cross. The chromosome intervals harboring these quantitative trait loci (QTLs) have also been linked to experimental autoimmune encephalomyelitis (EAE),[36] collagen-induced arthritis (CIA),[17] classical adjuvant arthritis (AA)[37] or pristane-induced arthritis (PIA).[38] They appear therefore to harbor genes of general importance for inflammatory diseases, and may in fact correspond to the recently described clusters of candidate susceptibility loci in autoimmune diseases.[39] We suggest that SIA is particularly suitable to identify susceptibility genes within these rat QTLs since provocation with endogenous squalene is pure and simple compared to the more complex provocations used to trigger other experimental diseases. Interestingly, we have recently produced congenic strains for *OiaW* and *Oia2* on chromosome 4 and *Oia3* on chromosome 10, and observed that these intervals do in fact confer susceptibility or resistance to SIA. The identification of susceptibility genes in these congenes is the focus of current studies.

In conclusion, arthritis induced with the cholesterol precursor squalene shares notable similarities with rheumatoid arthritis, and raises interesting questions concerning the role of endogenous molecules with adjuvant properties in chronic inflammatory diseases.

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