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# Arthritis induced in rats with adjuvant oil is a genetically restricted, αβ T-cell dependent autoimmune disease

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## SUMMARY

Adjuvant arthritis in rats is usually induced by injection of mycobacterium tubercle cell walls suspended in various adjuvant oils such as Freund's incomplete adjuvant (FIA) or pristane. We have recently shown that injection of adjuvant oils without inclusion of mycobacterium tubercle cell walls triggers arthritis [oil adjuvant-induced arthritis (OIA)] in the DA rat strain. The OIA is a genetically restricted disease since only DA rats are susceptible while Lewis, DA-fostered Lewis and $F_1$ (Lew × DA) rats are relatively resistant. Activated αβ T cells infiltrate the affected joints of adjuvant oil-injected DA rats and treatment with monoclonal antibodies to the αβ T-cell receptor abrogates development of arthritis. These findings show that αβ T-cell activation is a critical event in the development of OIA.

## INTRODUCTION

As models for rheumatoid arthritis (RA), severe polyarthritis can be induced in rats after injection of various adjuvants or after immunization with type II collagen (CII).[1,2] The adjuvant arthritis (AA) model is usually induced by a single injection of Freund's complete adjuvant (FCA), i.e. mycobacterial cell wall antigen suspended in mineral oil. Most rat strains are susceptible to adjuvant arthritis induced with FCA[3] and this model has for some time been the subject of intense investigation regarding its nature and usefulness as a model for RA. Recently, these investigations have focused on possible antigen-specific cross-reactions between tubercle cell wall antigens and various autoantigens such as cartilage proteoglycans and 65,000 MW heat-shock protein.[4] It has not, however, been possible to isolate an arthritogenic immunogen (i.e. an antigen eliciting an immune-specific response) in the tubercle cell wall fraction. Instead the minimal arthritogenic structures have adjuvant (i.e. immune stimulatory) but not immunogenic properties.[5] The synthetic analogue of the minimal arthritogenic structure derived from bacterial cell walls is muramyl dipeptide (MDP) which induces severe arthritis but does not give rise to a specific immune response.[6,7] Moreover, another synthetic adjuvant, lacking immunogenic properties, has been described which possesses arthritogenic capacity when added to mineral oil; CP-20,961 [*N*,*N*-dioctadecyl-*N'*,*N'*-*bis*(2-hydroxyethyl)propanediamin].[8,9]

It has been suggested that adjuvant enhances immune response by acting as a surface-active agent leading to activation of antigen-presenting cells.[10] Adjuvant structures are contained in certain synthetic oil compounds such as pristane, and in Freund's incomplete adjuvant (FIA), being a mixture of paraffin oils (85%) and mannid-oleat (15%). Within 1 hr after injection the adjuvant oil disseminates to draining lymph nodes where it exerts its immunostimulatory effect.[11] A common feature of arthritogenic adjuvants is their tendency to induce inflammatory granulomas and to stimulate the T-cell compartment.[8,12] It has previously been suggested that adjuvant activates autoimmune responses to endogenous antigens such as CII[9,13,14] although in other studies no response to CII could be recorded.[15–18] However, there are many obstacles towards establishing a possible relationship between arthritis and an immune response to a putative autoantigen. There is for instance no evidence yet that adjuvant arthritis in rats, induced with adjuvant oils or with CP-20,961, i.e. in the absence of an exogenous immunogen, is mediated by T lymphocytes or is genetically restricted as should be expected from an immune-specific autoimmune disease. Furthermore, it is not easy to provide clear evidence for a specific autoimmune response since not even in arthritic models induced with specific joint antigens[19,20] has a critical cell or an autoantigenic structure yet been isolated. We have previously described that DA but not Lewis rats develop a chronic form of arthritis after immunization with CII emulsified in adjuvant oil[21,22] and have recently shown that

Abbreviations: AA, adjuvant arthritis; CIA, collagen-induced arthritis; CII, type II collagen; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; MHC, major histocompatibility complex; OIA, oil-induced arthritis; RA, rheumatoid arthritis; TcR, T-cell receptor.

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the DA strain also develops arthritis after injection with adjuvant oil only.[23,24] In this study we describe the genetic restrictions, immunohistopathology and T-cell dependency of the oil-induced arthritis which show that the disease is dependent on endogenously activated and presumably autoreactive $\alpha\beta$ T cells critical for development of an acute inflammation of the joints.

## MATERIALS AND METHODS

*Induction and evaluation of arthritis*

DA rats, originally obtained from Bantin and Kingman Ltd, N. Humberside, U.K. and Lewis rats, originally obtained from Moellegaard Labs, Roskilde, Denmark, were bred and kept at the Biomedical Center in Uppsala, Sweden. In a separate experiment specific pathogen-free DA and Lewis rats were obtained from ZFV (Zentralinstitut für versuchstierzucht, Hannover, Germany) and kept isolated at the animal department of Institution for Pathology, Uppsala University, Sweden. The rats were kept in a climate-controlled environment with 12 hr light/dark cycles, housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum*. All experiments were performed on rats aged 8–12 weeks which were age- and sex-matched before the experiments. During the experiments two to three rats were housed in each cage. The rats were screened for pathogens and found to be free from common pathogens including Sendai virus, Hantaan virus, coronavirus, reovirus, cytomegalovirus and mycoplasma pulmonis. Native rat CII was prepared from a rat chondrosarcoma either with pepsin digestion or from lathyritic chondrosarcoma as described previously.[25] For induction of CIA native CII were dissolved in 0·1 M acetic acid 4° and emulsified 1:1 on ice with FIA (Difco, Detroit, MI) to a final concentration of 0·5 mg/ml. Rats were injected intradermally in the base of the tail with 300 $\mu$l of the emulsion. For induction of adjuvant arthritis either various adjuvant oils such as mineral oil (FIA; Difco) and pristane (2,6,10,14-tetramethylpentadecane) (Aldrich Inc, Milwaukee, WI) inducing oil adjuvant arthritis, or adjuvants solubilized in FIA such as CP-20,961[8] and *Mycobacterium butyricum* (=FCA) were used. CP-20,961 was generously supplied by Dr Belcher (Pfizer Inc., Groton, CT). When using FIA, FCA or pristane, 150 $\mu$l was injected intradermally in the base of the tail. In the case of CP-20,961 the compound was solubilized in FIA to a concentration of 50 mg/ml and 150 $\mu$l of the solution was injected intradermally in the base of the tail. Arthritis development was followed by a macroscopic scoring system for the four paws ranging from 0 to 3 (1 = swelling and/or redness of one toe or finger joint; 2 = two or more joints involved; 3 = severe arthritis in the entire paw). The mean severity is calculated only from rats with arthritis. Rats induced with CII/FIA, FIA and FCA were terminated 7 weeks after induction and rats induced with pristane and CP-20,961 were terminated 3 weeks after induction. The right limbs from four DA rats with oil-induced arthritis (OIA) and from two normal DA rats were demineralized, frozen in isopentane, cryosectioned and subjected to immunohistochemical analyses using techniques which have been earlier described.[26,27] As primary reagents were used antibodies to $\alpha\beta$ T-cell receptor (R73),[28] CD4 (W3/25),[29] CD8 (OX8),[30] major histocompatibility complex (MHC) class II (OX6),[31] sialoglycoprotein on neutrophils, some macrophages and T cells (W3/13),[29] interferon-gamma[32] and rat interleukin-2 (IL-2) receptor (ART18).[33] The relative frequency of positively stained cells in the synovium/pannus tissue was estimated: 0% (−); <0·5% (+); 0·5–5% (++); 5–20% (+++); 20–50% (++++).

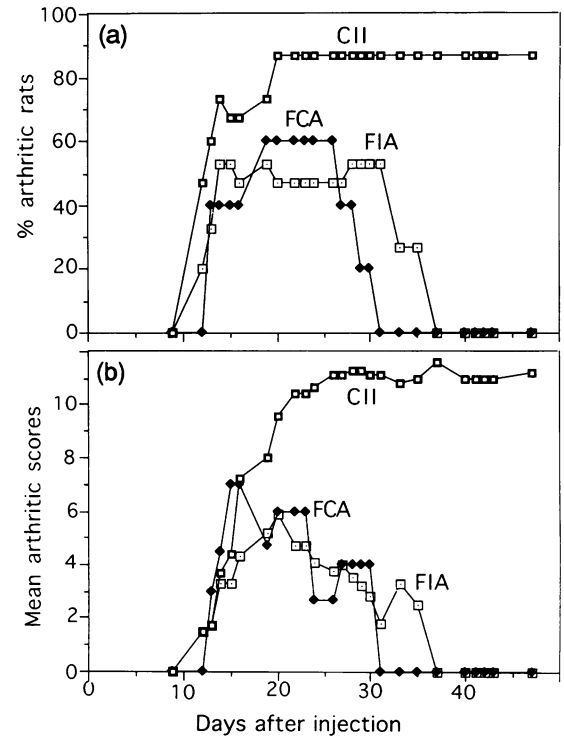


**Figure 1.** Development of arthritis induced with FIA (=OIA) ($n$=15), FCA (adjuvant arthritis) ($n$=5) and homologous CII (CIA) ($n$=15). (a) Frequency of arthritis; (b) mean severity of arthritis among rats with arthritis.

*Antibody treatment*

The hybridoma R73, producing mouse anti-rat $\alpha\beta$ TcR antibodies, was a kind gift of Dr T. Hünig, München, Germany.[28] Hy2.15, a mouse IgG1 which recognizes tri-nitrophenyl phosphate (TNP) was used as a control antibody. The antibodies in hybridoma supernatants were affinity purified on Protein A-Sepharose (Pharmacia AB, Uppsala, Sweden), dialysed with phosphate-buffered saline (PBS) and sterile filtered. The protein content was quantified by absorbance measurement at 280 nm. Five hundred micrograms was administered intraperitoneally on Days 14, 15, 20 and 21 after FIA injection.

## RESULTS

### Disease course of OIA and homologous CII-induced arthritis (CIA) in DA rats

DA rats develop severe arthritis after a single intradermal injection of FIA or with pristane, a synthetic oil (OIA) (Fig. 1, Table 1). In contrast, olive oil is ineffective. Macroscopic visible arthritis appears suddenly 10–12 days after injection of adjuvant oil and reaches an incidence of 50–100%. Swelling and erythema are first seen to affect the hind paws and usually also spread to the front paws. The disease course is acute and subsides 2–3 weeks after onset. A similar disease course is seen after injection

**Table 1.** Induction of arthritis in DA rats by different treatments

| Treatment | Model† | *n* | Incidence (%) | Maximal mean severity | Disease course |
|---|---|---|---|---|---|
| PBS | Control | 5 | 0 | | |
| Olive oil | Control | 5 | 0 | | |
| FIA (from Difco) | OIA | 5 | 60 | 9·8 | Acute |
| FIA (from Sigma) | OIA | 5 | 100 | 8·1 | Acute |
| Pristane | OIA | 16 | 69 | 7·5 | Acute |
| FIA/CP-20,961 | AA | 14 | 100 | 12 | Acute* |
| FCA | AA | 5 | 60 | 7·0 | Acute |
| FIA/rat CII in acetic acid | CIA | 12 | 100 | 10·0 | Chronic |
| FIA/acetic acid | OIA | 5 | 60 | 7·0 | Acute |
| FIA/ovalbumin | Control | 5 | 0 | | |

* Based on studies by Chang *et al.*[6] In our studies the experiment was ended a few days after onset to reduce the suffering of the rats since the disease is very severe.

† OIA, oil-induced arthritis; AA, adjuvant arthritis; CIA, collagen-induced arthritis.

**Table 2.** Genetic restriction of adjuvant oil-induced arthritis

| Rat | *n* | Treatment | Incidence (%) | Maximal mean severity |
|---|---|---|---|---|
| Lewis (DA-fostered)* | 10 | FIA | 0 | |
| DA (DA-fostered)* | 9 | FIA | 55 | 4·5 |
| Lewis | 5 | FIA | 0 | |
| Lewis | 10 | pristane | 0 | |
| Lewis | 10 | CP-20,961 | 100 | 12·0 |
| $F_1$ (DA × Lewis) | 5 | FIA | 0 | |
| $F_1$ (DA × Lewis) | 9 | pristane | 0 | |
| $F_1$ (DA × Lewis) | 9 | CP-20,961 | 100 | 12·0 |
| Lewis (SPF)† | 5 | FIA | 0 | |
| DA (SPF)† | 5 | FIA | 80 | 6·0 |

* One-day-old neonate DA and Lewis rats were randomly mixed and raised by DA mothers. They could later be easily separated by their different coat colours.

† Specific pathogen-free (SPF) rats from ZFV.

**Table 3.** Summary of immunohistochemical analyses of serial sections from arthritic joints

| Rats (no. of investigated rats) | Immunohistochemical stainings | | | | | |
|---|---|---|---|---|---|---|
| | MHC class II (OX6) | Neutrophils (W3/13) | CD4 (W3/25) | CD8 (OX8) | αβ TcR (R73) | CD25 (IL-2R) (AR18) |
| Normal (2) | + | + | + | − | − | − |
| OIA (4) | +++ | ++++ | ++ | + | ++ | + |
| CIA (4) | +++ | +++ | ++ | + | ++ | + |

of FCA containing mycobacterial cell wall fragments whereas addition of the synthetic adjuvant CP-20,961 in FIA induces a more severe arthritis with an earlier onset (Day 9). Addition of ovalbumin, a potent heterologous immunogen, to FIA abrogates the development of arthritis. In contrast, addition of rat CII, an autoimmunogen, to FIA produces a severe chronic arthritis known as CIA.[22]

The OIA disease is specifically located to joints since histopathological analysis of other organs, including salivary glands, thyroid gland, kidneys, spinal cord, skin and stomach, were negative.

## OIA is genetically restricted

The Lewis strain, which is one of the most commonly used inbred strains for studies of adjuvant arthritis induced with

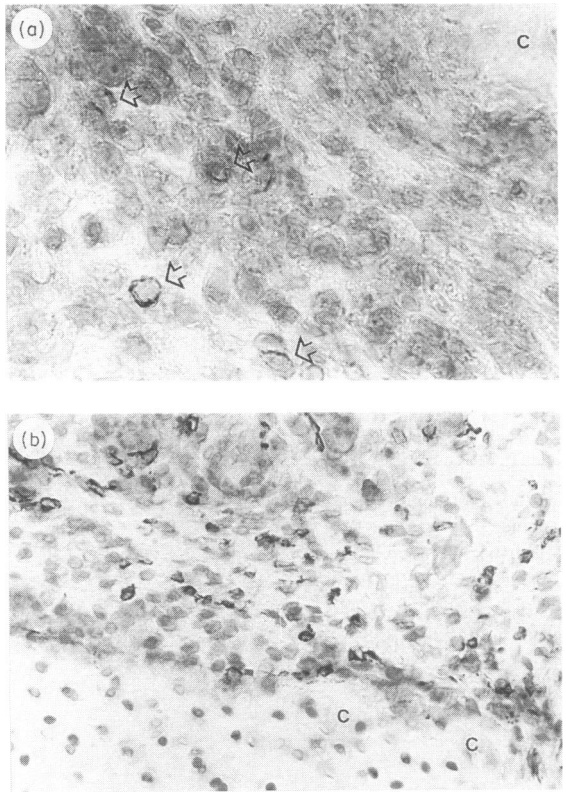


**Figure 2.** Examples of immunohistochemical stainings of an arthritic joint from a rat with OIA. (a) Staining with antibodies to $\alpha\beta$ TcR (R73) showing positively stained lymphocyte-like cells in pannus tissue close to cartilage ( $\times$ 280); (b) staining with antibodies to MHC class II (I-A) molecules staining cells within pannus tissue close to cartilage.

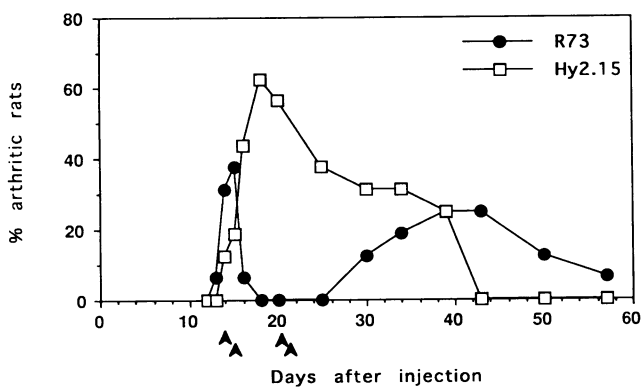


**Figure 3.** Development of OIA induced with FIA when treated with antibodies to $\alpha\beta$ TcR (R73) presented as frequency of arthritic rats. Arrows indicate injections of anti-TcR (16 rats) or control (Hy2.15) (16 rats) antibodies, 500 $\mu$g/rat Days 14, 15, 20 and 21.

FCA, is more resistant to OIA than DA (Table 2). The differences of susceptibility to OIA in our DA and Lewis rats can be due to both environmental and genetic influences as has been shown for other arthritic models in rats.[34-37] The two strains have been bred and kept in the same animal room and under identical conditions. Screening for occurrence of common pathogens was negative and did not reveal any differences between the two strains. In a separate experiment the susceptibility to OIA in DA rats and resistance in the Lewis rats was confirmed with specific pathogen-free rats obtained from ZFV (Zentralinstitut für versuchstierzucht). Moreover, our Lewis rats are capable of developing arthritis since induction with CP-20,961 in FIA was effective (Table 2). To exclude the possibility of a neonatally transmitted infectious disease in our DA rats we compared OIA susceptibility of DA and Lewis rats fostered by DA mothers from Day 2 after birth. Only the DA rats developed OIA. $F_1$ hybrid rats between Lewis and DA were not susceptible to OIA but were capable of developing arthritis after induction with CP-20,961 in FIA. These experiments suggest that the susceptibility of DA rats to OIA are genetically determined and dependent on recessive genes in the DA or dominant suppressive genes in the Lewis strain.

### A critical role of $\alpha\beta$ TcR expressing T cells in the development of OIA

In both OIA and CIA a severe and erosive arthritis develops. Immunohistopathological analysis of the acute phase of arthritis indicates a similar type of inflammation as has been described for CIA induced with heterologous CII.[26,27] In both OIA and CIA induced with homologous CII, few but significant numbers of activated $\alpha\beta$ T cells are present in the inflamed synovia as showed with immunohistochemical stainings using antibodies to $\alpha\beta$ *T*-cell antigen receptor and IL-2 receptors (Table 3, Fig. 2). In addition, in both CIA and OIA an enhanced expression of MHC class II molecules was seen. To assess whether $\alpha\beta$ T cells play a functional role in the development of OIA as has been shown for the development of CIA[38] we treated the rats with anti-$\alpha\beta$ T-cell receptor (R73) antibodies. Injection of R73 antibodies at Days 12–20 after the FIA injection completely reversed arthritis in rats that had already developed arthritis, while rats which had not yet developed arthritis when R73 treatment was commenced either never acquired the disease or developed a delayed onset of arthritis (Fig. 3). The effect was seen within 1 day of the first injection suggesting that T cells play a potent and critical role in disease development.

## DISCUSSION

Induction of arthritis with adjuvant oils such as FIA or pristane (=OIA) provides possibilities to address the question whether non-immunogenic adjuvant promotes arthritis by non-specifically activating the reticulo-endothelial system, which is richly present in the synovial tissue, or whether the immune system is critically involved. The OIA model is well suited to answer these questions since no exogenous immunogen such as bacterial antigens is introduced. Therefore, it is not necessary to relate our findings to the recently highlighted proposals of immunogenic cross-reactions between mycobacteria heat shock proteins and cartilage proteoglycans or endogenous heat-shock proteins as the cause of adjuvant arthritis.[4] Moreover, genetic studies of FCA-induced adjuvant arthritis have proved to be difficult because of the strong environmental influence of bacterial infections on the rats.[35]

The OIA is not dependent on any exogenously added immunogen and is therefore a purer model for studies of whether immune recognition of endogenous antigens are critical

in the development of arthritis. In addition, the pronounced susceptibility to OIA in the DA compared with Lewis rats seems to be genetically rather than environmentally determined. The disease susceptibility is influenced by recessive genes in DA or alternatively dominant suppressive genes in the Lewis rats. The different susceptibility to OIA between DA and Lewis rats provides the possibility to clarify the influence of specific genes of importance for adjuvant arthritis with no disturbance of exogenously introduced immunogens. In this context it is of interest that so far the only published evidence for a MHC influence on FCA-induced adjuvant arthritis has suggested that susceptibility to adjuvant arthritis is more closely related to the $RT1^a$ haplotype, carried by DA, and not to the $RT1^l$ carried by F344 and also by Lewis.[39]

Two findings in the present investigation suggest that development of OIA is critically dependent on the activation of autoreactive T cells. First, a few but significant number of activated T cells, expressing IL-2 receptors and $\alpha\beta$ TcR, infiltrate the arthritic joints of FIA-injected DA rats. Second, *in vivo* treatment with antibodies against $\alpha\beta$ TcR abrogates development of OIA. A critical role for $\alpha\beta$ TcR-expressing T cells has recently been demonstrated also in adjuvant arthritis induced with tubercle antigens[40] and in CIA induced with homologous CII.[38] The present finding that OIA in DA rats is an inflammatory disorder primarily affecting the joints suggests that the adjuvant triggers a local inflammatory response involving autoreactive T cells.

Important questions arise on how, where and with which specificity such autoreactive and arthritogenic T cells are activated. At present we favour the possibility that the adjuvant oil, after injection, rapidly spreads in the body and induces a generalized activation of antigen-presenting cells in the immune system and macrophages in the reticuloendothelial system including synovial macrophages. The antigen-presenting cells, which naturally carry self peptides bound to their MHC class II molecules,[41] will activate autoreactive T cells by providing them with co-stimulatory signals. Armed with this view it is possible to explain the observed abrogating effect on arthritis development by addition of ovalbumin in the adjuvant; this will lead to blockage of self peptide presentation by ovalbumin peptides and therefore lead to an anti-ovalbumin response instead of an arthritogenic autoreactive response. In the case of addition of rat CII to the adjuvant oil an immune response to a cartilage-specific autoantigen will develop with an eventual development of CIA instead of OIA. However, we do not propose that OIA is necessarily dependent on the activation of CII-reactive T cells although a comparison with the CIA model might be fruitful especially since the same rat strain (DA) is highly susceptible for both diseases. For such a comparison it is important to address three main topics: the cause of the chronicity in the CIA compared with the acute disease in OIA; the role of MHC which is clearly of major importance in CIA[22] but not yet investigated in OIA; the role of CII autoimmunity which develops differently in the two models.

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