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Pristane-Induced Arthritis in Rats

A New Model for Rheumatoid Arthritis with a Chronic Disease Course Influenced by Both Major Histocompatibility Complex and Non-Major Histocompatibility Complex Genes

Carina Vingsbo,* Pernilla Sahlstrand,* Johan G. Brun,† Roland Jonsson,† Tore Saxne,‡ and Rikard Holmdahl*

From the Department of Cell and Molecular Biology, Section for Medical Inflammation Research, and the Department of Cell and Molecular Biology,‡ Section for Connective Tissue Biology, Lund University, Lund, Sweden, and the Broegelmann Research Laboratory for Microbiology,† University of Bergen, Norway*

We present a novel animal model for rheumatoid arthritis induced with a well defined synthetic adjuvant oil, pristane. Two weeks after a single intradermal injection of 150 μl of pristane, the rats developed severe and chronic arthritis. The inflammation was restricted to the joints and involved pannus formation, major histocompatibility complex (MHC) class II expression, and T lymphocyte infiltration. The initial development as well as the chronic stage of pristane-induced arthritis was ameliorated by treatment with antibodies to the αβ-T-cell receptor showing that the disease is T cell dependent. Increased levels of interleukin in serum was seen after pristane injection but not during the chronic stage of arthritis. Joint erosions were accompanied by elevated serum levels of cartilage oligomeric matrix protein. Comparison of MHC congenic LEW strains showed that the severity and chronicity of arthritis varied among the different MHC haplotypes. Rats with RT1^f haplotype showed a significantly higher susceptibility to pristane-induced arthritis. A strong influence of non-MHC genes was also suggested by the variability of arthritis susceptibility among different strains with the same MHC haplotype; the most susceptible background was the DA and the least susceptible was the E3. Arthritis induced with a well defined nonimmunogenic adjuvant, with a disease course that closely resembles that of rheumatoid arthritis, makes a suitable animal model for future studies of the pathology and genetics of rheumatoid arthritis. (Am J Pathol 1996, 149:1675–1683)

Rheumatoid arthritis (RA) is an autoimmune disease that is dependent on both genetic and environmental factors. Experimental animal models in rats and mice have proven to be useful in the studies of the autoimmune mechanisms of RA. Arthritis can be induced in rats by an intradermal injection of *Mycobacterium tuberculosum* suspended in mineral oil.[1] This leads to severe arthritis, but the disease is not restricted to the joints and the severity of the arthritis does not progress.[2] The introduction of bacterial antigens give rise to a strong bacteria-specific immune response that will perturb the immune system of the rat and lead to systemic adjuvant disease.[2] An alternative model of RA is the induction of arthritis in the DA rat with Freund's incomplete adjuvant.[3,4] This model does not require foreign immunogenic material, but the subsequent disease is not chronic, which limits its use as a model for RA. Arthritis can also be induced in rats with the synthetic adjuvant avridine dissolved in Freund's incomplete adjuvant.[5] We

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Address reprint requests to Dr. Rikard Holmdahl, Department of Cell and Molecular Biology, Section for Medical Inflammation Research, Lund University, Box 94, 22100 Lund, Sweden.

have shown that this disease, in accordance with RA, is T cell dependent and that the chronicity of arthritis is influenced by major histocompatibility complex (MHC) genes.[6] Immunization with cartilage specific proteins induce arthritis in mice and rats. The type II collagen (CII)-induced arthritis (CIA) is a useful model for studies of MHC association and autoimmune recognition of a specific autoantigen (reviewed in Ref. 7). However, neither CII nor any other cartilage-specific antigen has yet proven to be of critical importance as an autoantigen in RA.

For the studies of the genetic association of autoimmune arthritis, it should be informative to use an animal model that is not dependent on the administration of exogenous antigens. An adjuvant arthritis model induced with a well defined adjuvant without immunogenic properties may be suitable, provided it mimics the development of RA. Of the arthritis models described so far, the avridine-induced arthritis in rats most closely meets these criteria. Problems with this model are, however, that the arthritis is very severe and that it is induced with a mixture of avridine and mineral oil. The mineral oil is a complex and poorly defined oil mixture that by itself has arthritogenic properties. In this study, we tested the well defined synthetic mineral oil pristane (2,6,10,14-tetramethylpentadecane).[8] Pristane is often used for producing ascites in mice and has earlier been shown to induce arthritis in mice,[9] with interesting immunological and genetic features.[10,11]

The arthritogenic properties of pristane in rats was found to fulfill the criteria we need for analyzing the genetic contribution to the development of autoimmune arthritis. We suggest that the pristane-induced arthritis (PIA) model will add features of great importance for the understanding of the pathogenesis and genetics of human autoimmune diseases, such as RA.

Materials and Methods

Rats

Specific-pathogen-free rats DA, LEW, LEW.1A, LEW.1C, LEW.1D, LEW.1F, LEW.1N, LEW.1W, LEW.1WR2, LEW.1AR2, DXEA, DXEB, DXEC, DXER and E3 (originating from Zentralinstitut Für Versuchstierzucht, Hannover, Germany) were kept in conventional animal facilities in a climate-controlled environment with 12-hour light/dark cycles, housed in polystyrene cages containing wood shavings, and fed standard rodent chow and water *ad libitum*. All experiments were performed on age- and sex-matched rats at an age of 8 to 14 weeks. The rats were found to be free from common pathogens including Sendai virus, Hantaan virus, coronavirus, reovirus, cytomegalovirus, and mycoplasma pulmonis.

Induction and Evaluation of Arthritis

Arthritis was induced by an intradermal injection at the base of the tail of 150 μl of pristane (2,6,10,14-tetramethylpentadecane) (Aldrich, Milwaukee, WI). Arthritis development was monitored by a macroscopic scoring system for the four limbs ranging from 0 to 4 (1, swelling and redness of one joint; 2, two joints involved; 3, more than two joints involved; and 4, severe arthritis in the entire paw).

Immunohistopathological Analyses of Arthritic Joints

Paws from LEW rats with haplotype a in the MHC class II region were taken at arthritis onset, day 14 after induction, or in a chronic stage of arthritis, day 135 after induction, and were analyzed with histopathological and immunohistochemical techniques. The paws were fixed and demineralized.[12] Cryosections were subjected to immunohistochemical analyses using techniques as described.[13] Used as primary reagents were antibodies to α/β-T-cell receptor (R73),[14] to TCRVβ8.2 (R78), to TCRVβ8.5 (B73), and to TCRVβ10 (G101.53),[15] CD4 (W3/25),[16] CD8 (OX8),[17] MHC class II (OX6),[18] sialoglycoprotein, CD43, on neutrophils, some macrophages and T cells (W3/13),[16] rat interleukin (IL)-2 receptor (ART18),[19] and complement receptor CR3 (CD11b) OX42.[20]

Antibody Treatment

LEW.1A and LEW.1W rats were treated with intraperitoneal injection of 500 μg of purified R73 antibody in phosphate-buffered saline (PBS) at days 12, 13, 19, and 20 after the pristane injection or in a late stage of arthritis, days 59, 66, and 67. Control rats were injected with pristane at the same time and treated with PBS only at the same time points.

Serum Samples

Serum was obtained by cutting the tip of the tail. The samples were stored at −70°C until assayed.

IL-6 Bioassay

The presence of IL-6 in the sera was determined with the B9 cell assay as described previously.[21] Briefly,

Table 1. *Susceptibility to Pristane-Induced Arthritis in Different Rat Strains*

Strain	RT1	Number of rats	Frequency of arthritis	Mean day of onset	Mean maximal score	Frequency of chronic arthritis
LEW	l	11	82%	23 ± 10	9	82%
LEW.1A	a	34	82%	28 ± 21	6	68%
LEW.1C	c	16	65%	22 ± 8	9	65%
LEW.1D	d	22	73%	26 ± 18	6	50%
LEW.1F	f	10	100%	17 ± 4	12†	100%*
LEW.1N	n	11	64%	21 ± 7	8	54%
LEW.1W	u	26	77%	22 ± 7	8	65%
LEW.1AR2	r3	23	70%	26 ± 14	8	60%
LEW.1WR2	r6	24	75%	25 ± 19	10	62%
DA	av1	17	100%	14 ± 2	11	100%
E3	u	23	9%	38 ± 6	2	0%
DXEA	av1	20	70%	31 ± 21	3	10%
DXEB	av1	24	33%	72 ± 34	5	30%
DXEC	u	20	10%	32 ± 15	3	0%
DXER	u	20	10%	34 ± 19	1	0%

This is a summary of five experiments with similar number of rats from the different strains. Chronic arthritis is defined as those having active arthritis day 90 or later after pristane injection.
*$P < 0.05$ compared with all other LEW congenic strains combined.
†$P < 0.001$ compared with all other LEW congenic strains combined.

B9 cells were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin, glutamine, 10% fetal calf serum, and supernatant from the IL-6-producing X63 cell line. Serum samples were diluted 1:25 in heat-inactivated medium as above. B9 cells were cultured with the serum samples for 72 hours and pulsed with [^{3}H]TdR for 4 hours. Cells were harvested in a Filtermate cell harvester (Packard Instruments, Meriden, CT). The incorporation of [^{3}H]TdR was determined in a Matrix 96 direct beta counter (Packard). One unit of IL-6 is defined as the amount inducing half-maximal proliferation of the B9 cell line (equivalent to ~2 pg/ml).

Cartilage Oligomeric Matrix Protein (Comp) Enzyme-Linked Immunosorbent Assay

Serum concentrations of COMP were determined by enzyme-linked immunosorbent assay using a slight modification of the assay for human COMP.[22] Rat COMP was prepared from rat chondrosarcoma as described.[23] The purified COMP was used for coating the microtiter plates and for preparing the standard curve included in each plate. The antiserum was raised in a rabbit against rat COMP and was a kind gift from Professor Mats Paulsson, University of Cologne, Germany. The intra-assay variability was less than 6%. All samples from one animal were run simultaneously on the same plate.

Statistics

The incidence was evaluated by χ^2 analysis, the mean maximal score with the Mann-Whitney U test, and the mean arthritis onset day with unpaired Student's *t*-test.

Results

Pristane-Induced Arthritis Is a Chronic Relapsing and Progressive Arthritic Disease

Susceptible rat strains developed severe arthritis with a sudden onset 2 to 3 weeks after pristane injection (Table 1). The arthritis usually started and persisted in the ankles of the hind paws in the LEW strains (Figure 1), whereas in the DA rats, arthritis more often started in the interphalangeal or metatarsal joints and later spread to the ankles. A score of 3 to 4 of a paw with an involved ankle joint corresponds to an increase in ankle thickness by 25 to 45%. The arthritis had both a relaps-

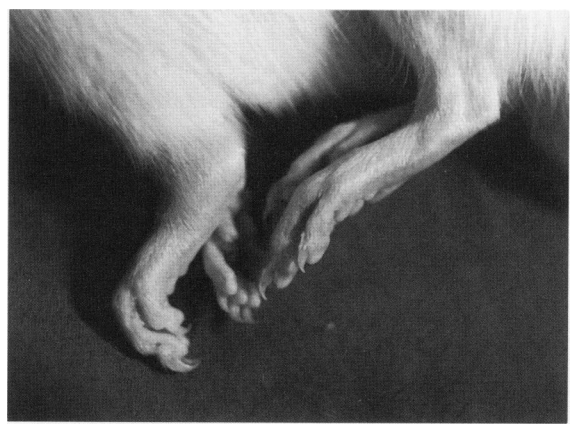

Figure 1. *Arthritic hind paw from a LEW.1A rat 122 days after pristane administration (left). To the right is a normal paw from an untreated rat.*

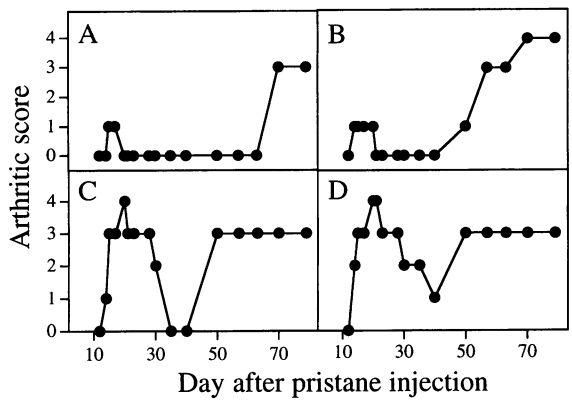

Figure 2. *Development of arthritis in a LEW1.WR2 rat showing each paw separately, exemplifying both a progressive and a relapsing disease course.* **A:** *Left front paw.* **B:** *Right front paw.* **C:** *Left hind paw.* **D:** *Right hind paw.*

ing and progressive disease course (Figure 2). Once arthritis had started in one joint, it could persist as an active lesion for a long time or it could decline in activity and later reappear. Both earlier affected joints and joints that had not been affected could suddenly develop arthritis along the disease course. The paws were gradually deformed, but excessive new bone formation was not as often seen as in adjuvant arthritis induced with *Mycobacterium tuberculosum*. An active arthritis was observed as long as the rats were monitored.

Histopathological examination of joints showed a deformed joint architecture (Figure 3). Synovial hyperplasia was the first morphological change to be observed and appeared in one DA rat at day 7 after pristane injection. By day 11, most rats had synovial hyperplasia and fibrin but no signs of clinical arthritis (Figure 3A). By days 15 to 19, joints with clinical arthritis showed a prominent pannus tissue (Figure 3B) containing neutrophils and large macrophage-like cells. Immunohistochemical staining of the joints showed a large number of MHC class-II-expressing cells and a few activated T cells expressing the IL-2 receptor (Table 2). A low but significant number of $\alpha\beta$-T-cell-receptor-expressing T cells were present showing a variable Vβ usage. Both $CD4^+$ and $CD8^+$ T cells were seen. The same extent of inflammatory infiltrate was seen in chronic arthritis (Table 2). The first signs of bone erosions were usually seen around day 15, ie, 2 days after clinical arthritis onset. It usually started subchondrally, and in joints with severe arthritis, the erosions spread along the periosteal surface. Cartilage was also eroded but mainly subsequent to severe subchondral bone erosions (Figure 3B). By day 122, the clinically affected joints were severely compromised by the erosions and cartilage was almost completely lost (Figure 3C). Histological examination of spleen, lymph node, pancreas, stomach, kidney, liver, muscle, testes, brain, and spinal cord from rats with chronic active arthritis did not reveal any abnormalities.

IL-6 Is Present in the Serum during the Acute Stage of PIA

Sera from DA rats injected with pristane were analyzed for the presence of IL-6 with the B9 cell line. All

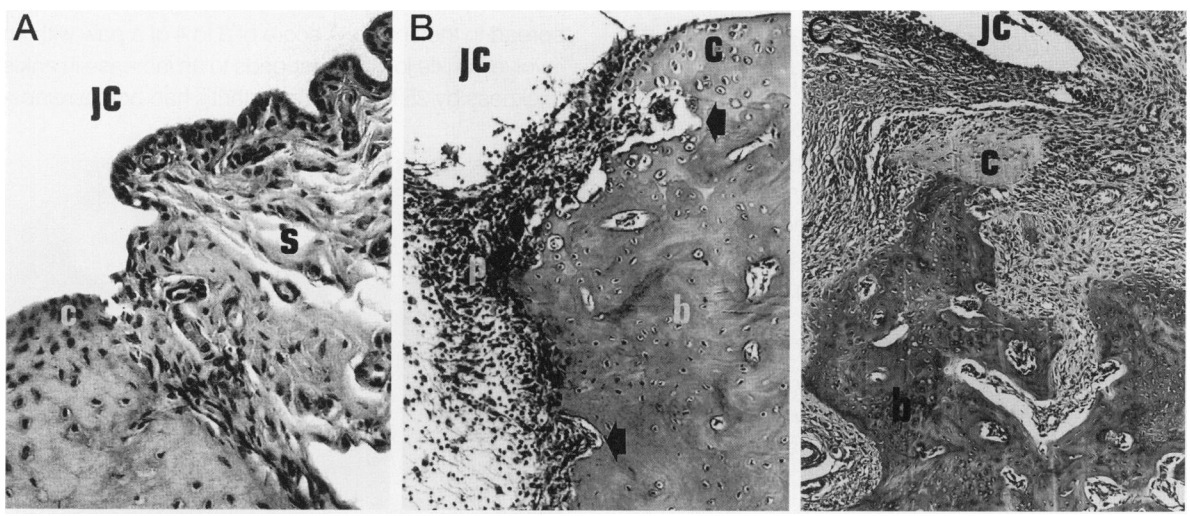

Figure 3. *Histopathological sections of DA rats at different stages of PIA development.* **A:** *Early morphological changes in the talocrural joint on day 11 after pristane injection showing synovial hyperplasia, increased vascularization, and fibrin precipitation. Original magnification, ×200.* **B:** *Pannus tissue and erosion of bone and cartilage in the talocrural joint on day 19. Original magnification, ×100.* **C:** *Chronic arthritis on day 122 showing massive infiltration of inflammatory cells and severe erosion of bone and cartilage. The entire joint surface has been engulfed in pannus, leaving only an island of cartilage at the collar of the joint. Original magnification, ×100. b, bone; c, cartilage; jc, joint space; p, pannus.* **Arrows** *denote erosions.*

Table 2. *Immunohistopathological Analyses of Arthritis*

	Clinical score	CD11b	CD43	MHC class II	CD4	CD8	αβTCR	TCRVβ8.2	TCRVβ8.5	TCRVβ10	CD25
Arthritis onset, day 14	4	++++	+++	+++	+++	+	++	+	+	+	++
	3	++++	++	+++	++++	++	++	−	+	+	++
Chronic arthritis, day 135	3	+++	+++	++	++	+	+	−	++	++	+
	3	+++	+++	++	++	+	+	++	++	+	−
No arthritis	0	++	−	+	+	−	−	ND	−	−	−

Two different rats were analyzed at each time point. The relative frequency of positively stained cells in the synovium/pannus tissue was estimated as 0% (−), <0.5% (+), 0.5 to 5% (++), 5 to 20% (+++), or 20 to 50% (++++). TCR, T cell receptor; ND, not done.
*The mean of three LEW rats injected with FIA or ovalbumin emulsified in FIA used as control rats and sectioned at day 21 after injection.

rats had increased serum levels of IL-6 at day 14 after pristane injection, which corresponds with the onset of arthritis. However, the time period of IL-6 production varied among the four rats analyzed. Only one of four rats showed increased IL-6 levels until day 49, although all rats still had active arthritis at this day (Figure 4). No detectable levels of IL-6 were observed in the PIA-resistant E3 strain during the same time period after pristane injection.

Cartilage Oligomeric Matrix Protein Levels Are Increased in the Sera of Rats with Newly Developed Arthritis

COMP is a cartilage-specific matrix protein, which consists of five subunits with M_r of ~100,000 linked together close to their amino termini to form a bouquet-like structure.[23] The protein shows extensive structural homologies with the thrombospondin family of proteins but is a unique gene product.[24] The function of the protein is unknown, but it shows a limited tissue distribution and is enriched in the superficial layer of cartilage. The serum levels of COMP were monitored during the disease course of arthritis in DA rats. Increased levels were seen 21 to 35 days after pristane injection but returned to normal by day 49. Initial values increased up to 80% in 9 of 13 arthritic rates (Figure 5). Of the 4 rats that did not show an increased level of COMP in the serum, 2 had low scores on day 100 (2 and 0). The time period of COMP elevation in serum thus correlated with early and severe erosions in the joints and is possibly predictive for a prolonged disease progression.

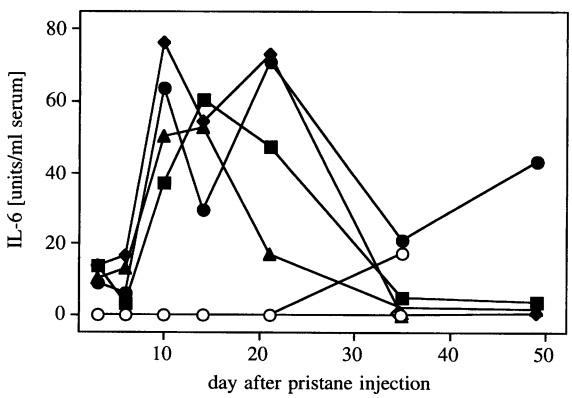

Figure 4. *Serum levels of IL-6 during the disease course of PIA.* Filled symbols *represent the four individual DA rats injected with pristane.* Open symbols *represent the two untreated rats.*

Susceptibility to Arthritis Is Influenced by Both MHC and Non-MHC Genes

There was a clear difference in arthritis susceptibility in the different rat strains studied (Table 1). The DA strain developed severe chronic arthritis in 100% of the animals with little variability in the disease onset

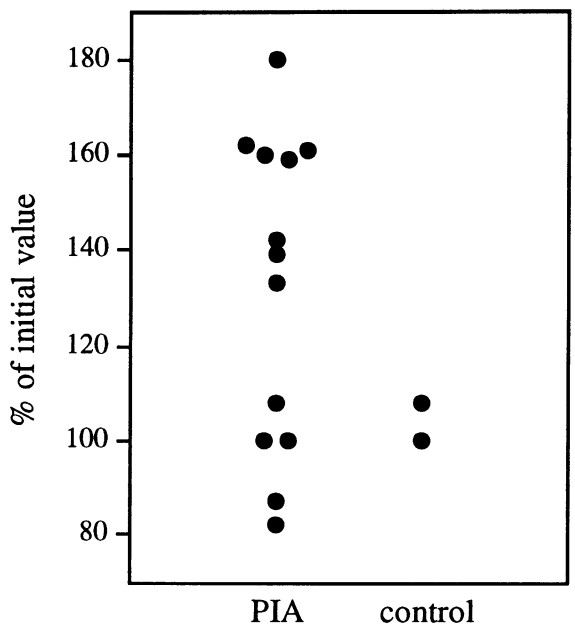

Figure 5. *Serum levels of COMP in DA rats on days 29 to 35 after pristane injection.*

Table 3. *Sex Differences in LEW Congenic Rats*

Sex	Number	Incidence	Mean maximal score	Mean day of onset
Females	91	91% $P < 0.01$	9 $P < 0.01$	20 ± 11 $P < 0.01$
Males	86	63%	6	31 ± 18

occurring around day 14. Injection of pristane intraperitoneally instead of subcutaneously also induced arthritis but with a later and more variable onset (data not shown). The E3 rat was relatively resistant with a mild and transient arthritis in only 2 of 23 animals. The LEW rat had an intermediate response with high incidence but with a late and variable onset. Four recombinant inbred strains, DXEA, DXEB, DXEC, and DXER, produced from DA and E3, showed a variable susceptibility to PIA (Table 1). Interestingly, only DXEA and DXEB, with the $RT1^{av1}$ haplotype from the DA rat were susceptible, whereas DXEC and DXER with the $RT1^{u}$ haplotype were resistant. To investigate whether this difference in susceptibility to PIA was associated with the MHC, we compared seven MHC congenic LEW strains. All of the RT1 haplotypes were responsive, but there were differences in the severity, incidence, and the time of onset of arthritis (Table 1). LEW.1F was exceptionally sensitive, with 100% of the animals developing chronic arthritis compared with 50 to 82% in the other strains ($P < 0.05$). The arthritis was also more severe in the LEW.1F strain ($P < 0.001$). The LEW.1A and LEW showed a higher incidence than the other strains. LEW.1D had the lowest frequency of chronic arthritis, 50%, a relatively mild disease, and a late onset.

Background genes clearly influenced the disease course of PIA. The DA background is highly permissive whereas the E3 genome is protective as seen by the reduced susceptibility in the recombinant strains DXEA and DXEB. Comparison of the LEW.1A and DA showed that the LEW background is permissive but with a reduced incidence and severity and a delayed onset. E3, DXEC, and DXER with the $RT1^{u}$ haplotype are relatively resistant to PIA. However, LEW.1W showed that the $RT1^{u}$ haplotype can be permissive to arthritis on the LEW background.

Females were in general more affected than males. In all rats with a LEW genetic background, a higher incidence, a higher severity, and an earlier arthritis onset were seen in females as compared with males (Table 3). A similar degree of female preponderance are seen in the avridine arthritis model and in CIA in rats.[25]

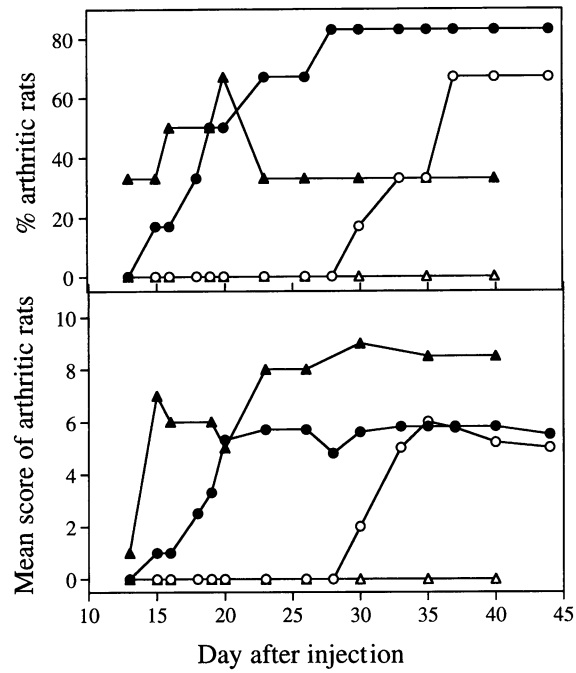

Figure 6. *T cell receptor blockage with the R73 monoclonal antibody* (open symbols) *during onset of arthritis in LEW.1A* (△) *and LEW.1W* (○) *or control treatment with PBS* (filled symbols).

Pristane-Induced Arthritis Is a T-Cell-Dependent Disease

Pristane is a nonimmunogenic adjuvant, and the arthritis may involve immune cells or it may be caused by a nonspecific triggering by the adjuvant. We therefore attempted to analyze the involvement to T cells. LEW.1A rats treated with antibodies to the $\alpha\beta$-T-cell receptor (the R73 antibody) at the expected period of onset did not develop arthritis (Figure 6). LEW.1W rats did develop arthritis after this treatment, but the onset was considerably delayed. This indicates that T cells are involved in the initiation of arthritis. To investigate whether T cells are of critical importance also in the chronic phase of disease, we treated arthritic LEW.1A rats with the R73 antibody at days 59 to 67 after pristane injection. A decrease in redness and swelling was observed, but at this time the paws were severely deformed and it was difficult to determine the effect of the treatment by clinical scoring. Histological analyses 8 days after the treatment showed a less active inflammation in the joints (Table 4). The pannus tissue decreased significantly ($P < 0.05$) after treatment, and in some individuals it disappeared completely. This implicates that autoreactive T cells, presumably specific for joint tissue antigens, may perpetuate the chronic inflammation in arthritis.

Table 4. *Chronic Arthritis after αβT-Cell-Receptor Blockage*

Rat	Treatment	Pannus	Periarticular inflammation
1	PBS	2	2.5
2	PBS	2.5	3.5
3	PBS	2.5	3.5
4	PBS	2.5	3.5
5	R73	1	2
6	R73	0	2
7	R73	0	1.5
8	R73	1.5	2.5
9	R73	2.5	2.5
10	R73	2	3.5
Mean	PBS	2.4	3.0
Mean	R73	1.2*	2.3

Each rat was morphologically evaluated from 0 (not detectable) to 4 (very severe).
*$P < 0.05$ *versus* control treatment with PBS only.

Discussion

PIA in the rat is a new animal model suitable for studies of basic mechanisms in the pathogenesis of RA. Similar to RA, it is a chronic active disease with erosions of peripheral joints, is associated with MHC genes, and is more susceptible in females than males.

PIA is induced by a single injection of pristane intradermally around the base of the tail. Sudden onset of arthritis appears 11 days or later after the injection. The disease is quite different from that of the mouse model induced with pristane in that no systemic abnormalities can be found and that the inflammation is restricted to the joints.

One of the most useful features of the PIA model is its pronounced chronic active disease course, which resembles RA. As in RA, cartilage and bone erosions are seen along the disease course, also at the chronic stages. The increase in serum levels of COMP coincided with early erosive changes in cartilage. The serum levels subsequently decreased. This is in line with the findings in human RA, for which increased serum COMP levels were found early in those patients who rapidly developed severe joint destructions, but at a later stage the COMP levels returned to normal.[26] A possible predictive value of serum COMP levels regarding the degree of future joint destructions in the rat model is also suggested by the finding of normal COMP levels in the two rats that had low arthritis scores at day 100.

PIA shares histological characteristics with other autoimmune arthritis models such as CIA and avridine-induced arthritis[6,27,28] as well as with RA. The chronic phase of disease has not yet been analyzed in the adjuvant arthritis induced with mycobacteria cell walls, simply because the disease is usually not active at late stages.[2] Analyses of chronic arthritis in PIA described herein show a pronounced bone and cartilage erosion. A prominent pannus tissue is present containing activated T cells, large macrophage-like cells, neutrophilic granulocytes, and class-II-expressing cells. In comparison with corresponding studies of human RA, the histopathology is strikingly similar but with one possible exception. In comparison with many,[29–31] but not all,[32] of the reports of human RA, the number of infiltrating T lymphocytes is higher in humans than in the rat. It is, however, shown that T lymphocytes are critical for arthritis development in animal models for RA.[4,6,33,34] In the present work, we show that αβ-T lymphocytes play a critical role both in the induction and in the chronic stage of PIA. This suggests that the pristane may trigger activation of autoreactive T lymphocytes, which permits an initial acute arthritis and, subsequently, a chronic and self-perpetuative attack on the joints.

As PIA is T cell dependent, it is of obvious interest that the chronicity and severity of PIA seems to be associated with MHC genes. Association with the $RT1^f$ haplotype, as seen in PIA, was also seen in avridine-induced arthritis[6] whereas CIA, induced with CII, is associated with $RT1^a$ and only weakly with $RT1^f$.[35] In addition, no antibody response to CII in PIA was detected (data not shown). Nevertheless, a common denominator may be the MHC class-II-restricted recognition of CII or possibly another so far unknown arthritogenic cartilage protein. PIA in mice shows a MHC association to the haplotypes $H\text{-}2^q$, $H\text{-}2^r$, and $H\text{-}2^d$.[11] Interestingly these haplotypes are also associated with CIA[36] and proteoglycan-induced arthritis.[37] The involvement of CII immune recognition in PIA in mice is further suggested by Rademacher and co-workers[38] who found that pretreatment of mice with pristane exaggerates the development of CIA and by Thompson et al,[39] who reported that mice tolerized to CII by oral administration have a reduced susceptibility to PIA.

Pristane is not known to have any immunogenic properties, and it is most unlikely that it would be immunologically recognized by T or B cells. Rather, the effects are more likely based on its capacity to nonspecifically act as an adjuvant. It is commonly used for the induction of ascites in mice and for transformation of murine B lymphocytes into lymphomas *in vivo*. The plasmacytoma-inducing capacity of pristane has been suggested to be dependent on IL-6.[40] IL-6 is an inflammatory cytokine also implicated in the pathology of several inflammatory diseases including RA, and it has been detected in the synovial fluid and sera of patients with RA.[41,42] In-

creased serum levels of IL-6 in murine and rat models of arthritis has also been reported.[43–45] We therefore investigated whether intradermal injection of pristane in the rat resulted in IL-6 production and whether this was a measure of arthritis-associated inflammation. We found dramatically increased serum levels of IL-6 after pristane injection. However, during the chronic stage of PIA, systemic IL-6 was not detected. We conclude that, in the case of PIA, IL-6 reflects the inflammatory response triggered by the adjuvant but is not necessarily connected with the development of chronic inflammation.

Taken together, we suggest that the PIA model will add features of great importance to the understanding of the pathogenesis and genetics of human autoimmune diseases, such as RA.

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