

# Immune Reactivity to Connective Tissue Antigens in Pristane Induced Arthritis

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**ABSTRACT.** *Objective.* Pristane induced arthritis (PIA) is a seropositive experimental murine model that closely resembles rheumatoid arthritis (RA). Immune reactivity to a broad spectrum of autoantigens has been recognized in this disease model. We investigated the specificity of the autoimmune response in PIA to determine whether reactivity to connective tissue antigens is associated with the development of arthritis.

*Methods.* DBA/1 mice were injected with pristane and evaluated for development of joint disease and autoimmunity. Lymph nodes, spleen, sera, and arthritic paws were investigated at 1, 2, 4, 6, 9, and 12 months postinjection. T cell responses to 16 different joint components were evaluated using proliferation assays, and sera were assayed by ELISA for antibodies to these joint antigens. Cytokine concentrations after antigenic stimulation were assessed by ELISA in cultured cell supernatants and by real-time polymerase chain reaction using mRNA from spleens and arthritic paws.

*Results.* ELISA revealed positive responses to glucose-6-phosphate isomerase, chondroitin sulfate B, collagen I, collagen II, aggrecan, and DNA between 4 and 12 months post-pristane injection. *In vitro* tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin 6 (IL-6) responses were detected during reactions to most antigens tested, while IL-4 responses were absent. Cytokine analysis in arthritic joints revealed consistent expression of IL-1, IL-4, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  mRNA.

*Conclusion.* These results indicate that PIA animals develop both T cell and antibody responses to a broad spectrum of connective tissue antigens. Biglycan, aggrecan, and decorin may be relevant antigens in the pathogenesis of PIA, but no specific reaction pattern could be associated with the occurrence of disease. The data suggest that the development of pristane arthritis is not dependent upon reactivity against a single connective tissue antigen, but is a polyspecific autoimmune response to joint components elicited in pristane injected mice. (J Rheumatol 2004;31:1497–505)

## Key Indexing Terms:

PRISTANE INDUCED ARTHRITIS AUTOIMMUNITY Th1 RESPONSE ANIMAL MODEL

Rheumatoid arthritis (RA) is a disease of unknown etiology characterized primarily by chronic synovitis, joint erosions, and a broad spectrum of immune abnormalities<sup>1</sup>. Serological abnormalities include the production of autoantibodies directed against immunoglobulins (rheumatoid factors)<sup>2,3</sup>, connective tissue components such as type II collagen (CII)<sup>4,5</sup>, cartilage proteoglycans, and DNA<sup>6,7</sup>. A T cell response has also been seen in response to CII, but only if a concurrent autoantibody against CII is present<sup>8</sup>. Patients with RA also have an increased response to heat shock protein 60 (HSP60) and HSP65<sup>9</sup>. Involvement of the cellular immune system is indicated by the immunogenetic association of RA with the histocompatibility antigen HLA-DR4<sup>10</sup>, and by the presence of abnormal immune cell subsets within the joint infiltrate<sup>11</sup>. Patients with RA have decreased

concanavalin A (ConA) reactivity as well as a decreased suppressor T cell population<sup>12</sup>. The development of autoimmunity in RA has been postulated to be antigen-driven. According to this hypothesis, an infectious agent or an antigenic element is presented in the context of class II molecules of the major histocompatibility complex (MHC) to a T cell population sequestered within the joint<sup>13</sup>. The T cell reactivity is predicted to crossreact with a joint component, causing amplification of the immune reaction and tissue damage. Simultaneous recruitment of additional mediators of inflammation may increase the range of the autoimmune response as cartilage derived antigens are exposed. However, studies of the regulatory lymphocyte subsets have identified no immune reactivity against an infectious agent or an antigenic element common to the development of RA, with the possible exception of a role for heat shock protein in arthritis development<sup>9,14</sup>. Flow cytometric analysis of synovial fluid (SF) lymphocytes has revealed decreased CD4/CD8 T cell ratios compared to peripheral blood, and increased expression of Class II MHC antigens (HLA-DR) on T cells<sup>15–17</sup>. The CD4+ T cells from these patients also have a depressed mitogen response<sup>18</sup>.

Pristane induced arthritis (PIA) is an excellent model to

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Submitted August 14, 2003; revision accepted February 4, 2004.

investigate the spectrum of autoimmune abnormalities seen in RA. The injection of pristane (2,6,10,14-tetramethylpentadecane) has been shown to induce a seropositive inflammatory arthritis in susceptible strains of mice<sup>19,20</sup>. The disease has been shown to occasionally remit and relapse<sup>21</sup> in a manner similar to RA. The unique value of the PIA model is its presentation as an inflammatory joint disease accompanied by hypergammaglobulinemia and a profile of autoantibodies including rheumatoid factor (RF), antibodies to HSP, and antibodies to collagen<sup>20,22</sup> that are induced in the absence of direct immunization. Autoimmunity to 60 kDa HSP is associated with the development of PIA, which lends credence to the environmental antigen theory of RA, as HSP60 concentrations increase with age and pristane injection causes arthritis after 4 months and in some cases as late 9 months postinjection<sup>23</sup>. Novel antigen responses are indicated by proliferative responses to protein extracted from PIA joints, but not to protein extracted from normal joints<sup>24</sup>. Mice also have decreased natural killer cell responses after pristane injection<sup>25</sup>, which may be related to a decrease in the lymphocyte population<sup>26</sup>. If the Th2 cytokines IL-4 and IL-10 are depleted, the severity of pristane arthritis may be decreased<sup>27</sup>. PIA is a CD4+ T cell-dependent disease, and the population of splenic lymphocytes must contain CD4+ cells in order to proliferate<sup>28</sup>. If CD4 T cells are depleted, PIA incidence and severity are greatly decreased, while CD8+ T cells are not crucial to disease development, although CD8+ cell depletion does cause RF levels to decrease<sup>29</sup>. It is also known that without bowel flora, both PIA and anti-HSP65 antibodies do not occur. If mice kept in a sterile colony are brought into a normal environment, they acquire bowel flora and anti-HSP65 antibody, and PIA susceptibility is restored<sup>30</sup>.

The pathogenesis of autoimmune arthritis is unknown, but both PIA and RA are postulated to be antigen-driven. We examined the evolution of antigen reactivity in PIA, and investigated the response to a variety of joint antigens over time to determine whether one dominant antigen is associated with joint disease. The role of the immunological attack on the joints in PIA was investigated by measuring *in vitro* proliferative responses, antibody responses to a variety of joint components, and the cytokine profile in affected paws, tissues and cell culture supernatants.

## MATERIALS AND METHODS

**Induction and assessment of pristane arthritis.** Female DBA/1 mice 6–8 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and quarantined in our facility for 2 weeks before experimentation. Then 0.5 ml pristane (Aldrich, St. Louis, MO, USA) was administered by intraperitoneal injection. Mice were weighed weekly and monitored for onset and progression of disease. Mice developing PIA were clinically assessed and paw measurements were recorded once a week until termination. An established arthritis scoring system<sup>31</sup> was used to evaluate disease: 0 = normal appearance and flexion, 1 = erythema and edema, 2 = visible joint distortion, 3 = ankylosis. Each limb was measured using a constant tension caliper (Dyer, Lancaster, PA, USA) and graded, giving a maximum

possible score of 12 per mouse. Mice were sacrificed at 1, 2, 4, 6, 9, and 12 months. After the 2 month timepoint, 6 or 7 of the 8 mice sacrificed from the pristane injected group at each timepoint had arthritis in one or more paws. Each sacrifice group was chosen by a randomized mouse number assignment before the start of the study.

**Connective tissue antigens.** Collagen type II (CII; provided by Dr. M. Griffiths, University of Utah), CII 245-270 peptide (the immunodominant peptide in collagen induced arthritis<sup>32</sup>; the generous gift of Dr. L. Myers, University of Tennessee, the chaperone protein BiP (provided by Dr. G.S. Panayi, University of London), aggrecan, biglycan, chondroitin sulfate, chondroitin sulfate B, keratin sulfate, decorin, fibronectin, glucose-6-phosphate isomerase (GPI; Sigma, St. Louis, MO, USA), joint extract from normal mice, joint extract from arthritic joints, DNA, and aggregated IgG (Ig) were used for proliferation assays, bulk tissue culture, and ELISA.

**Preparation of aggregated IgG.** Protein A (Sigma) was used for isolation of IgG from mouse sera. Pooled mouse sera were added to saturated ammonium sulfate (Sigma) and incubated overnight at 4°C. The precipitate was centrifuged for 1 h at 13,000 rpm and redissolved in a minimal volume of phosphate buffered saline (PBS). The solution was dialyzed overnight against PBS at 4°C with frequent changes of PBS, and then loaded onto a Protein A column (Sigma). After 10 min, the column was washed with PBS to remove all unbound protein, and immunoglobulin was eluted with 0.2 M glycine and redialyzed against PBS. Protein concentration was determined using the Pierce protein assay (Rockford, IL, USA) and adjusted to 2 mg/ml. The sample was then heated to 85°C for 40 min to facilitate aggregation.

**Normal and arthritic joint extract.** Joints were harvested from either arthritic or nonarthritic mice and the ankle and wrist tissue was dissected free of skin and muscle, and washed by immersion in saline. The tissue from whole joint was cut into small sections, and added to 3.0 ml of PBS. Proteins were extracted using a Polytron tissue homogenizer (Kinematica, Switzerland). Tissue was homogenized at low speed for 20 s and then at high speed for 20 s. The extract was clarified by centrifugation at 3000 rpm for 10 min to remove debris, then supernatant was transferred to new tubes and the protein concentration determined.

**Isolation of lymphocytes from spleens and lymph nodes.** Spleens and lymph nodes (mediastinal, lateral axillary, popliteal, and superficial inguinal) were removed from the mice and immediately immersed in PBS. Tissue was mechanically disrupted to release cells, which were suspended in 10 ml sterile PBS and centrifuged for 10 min at 1500 rpm. Lymph node cells were resuspended in RPMI (Gibco, Grand Island, NY, USA), and cell concentration was determined using a hemocytometer. Prior to resuspension in medium and counting, red blood cells were removed from the spleen preparations by adding distilled water for 10 s and then adjusting to isotonic conditions with 10× PBS. Spleen cells were then counted, washed, and resuspended in RPMI at a final concentration of  $2.5 \times 10^6$ /ml.

**In vitro stimulation with connective tissue antigens.** PIA and control mice were sacrificed to determine T cell responses to ConA and the battery of antigens. Lymph nodes and spleens were harvested and 1 ml of cell suspension ( $2.5 \times 10^6$ /ml) was cultured in 12-well tissue culture plates (Costar, Corning, NY, USA) with various antigens at 50 µg/ml in complete RPMI-1640 medium. Cells were incubated 3 days at 37°C in a 5% CO<sub>2</sub> atmosphere in the presence of antigen. Supernatant was then removed for cytokine analysis.

**Proliferation assays.** Cell aliquots (100 µl;  $2.5 \times 10^6$ /ml) from the bulk culture stock were transferred to 96-well tissue culture plates (Costar) with 50 µg/ml of each antigen in complete RPMI-1640 medium. Cells were incubated 72 h at 37°C in the presence of antigen. Then 20 µl of MTT (a mitochondrial enzyme substrate) solution (5 mg/ml; Sigma) was added per well. After 6 h, the culture supernatant was discarded, and 200 µl of 10% sodium dodecyl sulfate solution added to each well. After incubation at 37°C overnight, the optical density at 590 nm was read using a microplate photometer (Molecular Devices, Sunnyvale, CA, USA). The mean and OD values, which provide a measure of cell proliferation, were

recorded for each cell sample with respect to antigen stimulation. Antigen-specific responses were expressed as (OD590 [stimulated culture] – OD590 [spontaneous proliferation culture])/OD590 [spontaneous proliferation culture].

**Measurement of supernatant cytokine concentrations.** Supernatant was removed from bulk culture after 3 days' incubation with antigen and stored at  $-80^{\circ}\text{C}$  until ELISA were performed to determine cytokine levels. Briefly, ELISA plates (Nunc-Immuno plates, Nunc, Roskilde, Denmark) were coated with 50  $\mu\text{l}$  of 0.1 M  $\text{NaHCO}_3$  containing 0.5  $\mu\text{g}/\text{ml}$  purified anti-IL-4, anti-IL-6, anti-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), or anti-interferon (IFN- $\gamma$ ; Pharmingen, San Diego, CA, USA) at  $4^{\circ}\text{C}$  overnight. Plates were washed 3 times with PBS containing 0.05% Tween 20 (Sigma), and nonspecific binding was blocked by the addition of PBS containing 5% nonfat milk overnight at  $4^{\circ}\text{C}$ . Then 50  $\mu\text{l}$  supernatant was added along with a standard and incubated overnight at  $4^{\circ}\text{C}$ . Subsequently, the plates were washed 6 times in PBS containing 0.05% Tween-20 and incubated with biotinylated secondary antibody at  $37^{\circ}\text{C}$  for 1 h. Plates were washed 6 times and streptavidin-APK (Pharmingen) was added and incubated at room temperature for 40 min. The plates were then washed 6 times and developed for 40 min in the dark, using p-nitrophenyl phosphate (Sigma) as a chromagen substrate. The resulting optical density was measured at 405 nm using a UV-max spectrophotometer (Molecular Devices).

**Extraction of mRNA from pristane-arthritic joint cells.** Arthritic ankle joints were removed from mice at 4, 6, 9, and 12 months post-pristane injection. The joints were divided by sagittal dissection, dissected free of skin and muscle, and washed by immersion in saline. The tissue from each whole joint section was cut into smaller sections, added to 3.0 ml of RNazol (Gibco), and extracted using a Polytron tissue homogenizer (Kinematica). The tubes were then centrifuged at 3000 rpm for 10 min and the supernatant transferred to new tubes and RNA extracted immediately. Then 0.2 ml of chloroform (Sigma) was added and the tubes were shaken vigorously for 20 s. The samples were set on ice for 5 min, and then centrifuged at 12,000 g for 15 min. The aqueous layer was collected, an equal volume of isopropanol was added, and samples stored at  $-20^{\circ}\text{C}$  overnight. The following day, the RNA was centrifuged again and then washed with 70% ethanol and centrifuged again at 7500 g for 8 min. DEPC water was added for storage and the concentration was measured. The quality and quantity of all RNA from joints was determined spectrophotometrically (OD 260/OD 280).

**Real-time PCR.** Real-time reverse-transcript polymerase chain reactions (RT-PCR) were performed and the gene activity of IL-1, IL-4, IL-6, and TNF- $\alpha$  was examined. Total ribonucleic acid (RNA) from spleen cells taken directly from the mice and arthritic joints was extracted as described above. cDNA was reverse transcribed from 0.5  $\mu\text{g}$  of total RNA in a 20  $\mu\text{l}$  reaction mixture containing 1 $\times$  PCR buffer, 500  $\mu\text{M}$  each of dNTP, 0.5 U/ $\mu\text{l}$  of RNase inhibitor, 2.5  $\mu\text{M}$  random hexamers, 5.5 mM  $\text{MgCl}_2$ , and 1.25 U/ $\mu\text{l}$  of reverse transcriptase (Perkin-Elmer, Norwalk, CT, USA). The reaction mixture was incubated in a Thermal Cycler (Perkin-Elmer) at  $25^{\circ}\text{C}$  for 10 min,  $48^{\circ}\text{C}$  for 25 min, followed by  $95^{\circ}\text{C}$  for 5 min. Real-time PCR was performed according to manufacturer's instructions. To standardize target gene level with respect to variability in quality of RNA and cDNA, we used GAPDH transcripts, a housekeeping gene, as an internal control. Reaction mixtures of 25  $\mu\text{l}$  included 12.5  $\mu\text{l}$  of 2 $\times$  SYBR<sup>®</sup> Green Master Mix and target gene primer pairs (at 400 nM final concentration) and 2  $\mu\text{l}$  cDNA. The sequences of the primer cytokines (IL-1, IL-4, IL-6, and TNF- $\alpha$ ) were purchased from Clontech, Palo Alto, CA, USA. All reagents were from Perkin-Elmer/Applied Biosystems. The reactions were run in MicroAmp optical 96-well reaction plates with MicroAmp optical caps for 40 cycles ( $95^{\circ}\text{C}/15$  s,  $60^{\circ}\text{C}/1$  min) in the ABI Prism 7700 Sequence Detector (PE-Applied Biosystems, Foster City, CA, USA) and the fluorescent signals were recorded dynamically. Normalization and analysis of the reporter signals ( $\Delta\text{Rn}$ ) at the threshold cycle was recorded by built-in software, and target gene copies were calculated against regression of the standard curve.

**Measurement of serum antibody levels.** All mice were bled before pristane

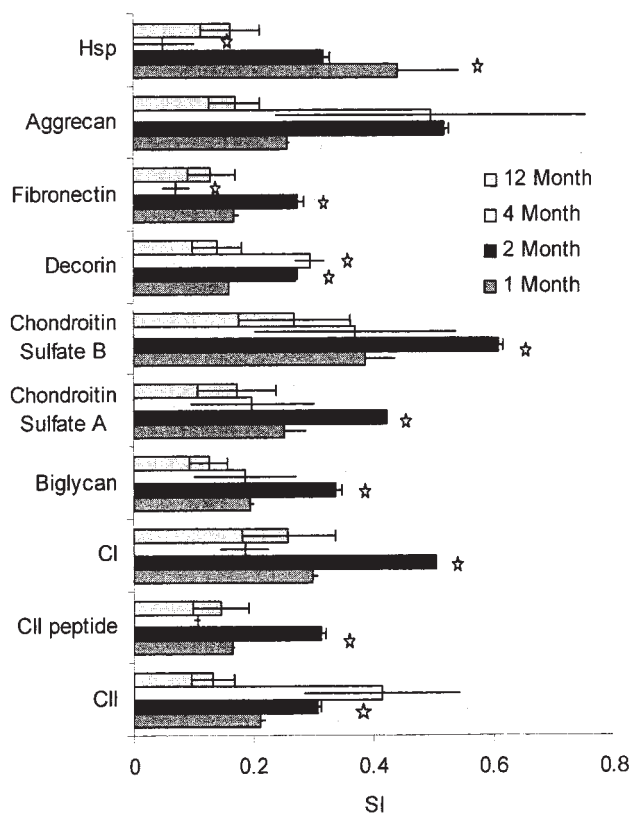
injection and monthly thereafter. Serum was separated from all samples and stored at  $-80^{\circ}\text{C}$ . Levels of antibodies were determined by ELISA as described<sup>33,34</sup>. Briefly, ELISA plates (Nunc-Immuno plates) were coated with 100  $\mu\text{l}$  coating buffer (0.4 M phosphate buffer, pH 7.6) containing 5  $\mu\text{g}$  of antigen, at  $4^{\circ}\text{C}$  overnight. Plates were washed 3 times with PBS containing 0.05% Tween 20 (Sigma) and nonspecific binding was blocked by PBS containing 5% nonfat milk overnight at  $4^{\circ}\text{C}$ . Mouse sera diluted 1/100 in 5% milk/PBS was added to each well and incubated overnight at  $4^{\circ}\text{C}$ . Then the plates were washed 6 times in PBS containing 0.05% Tween-20 and incubated with alkaline phosphatase conjugated goat anti-mouse Ig (Southern Biotechnology, Birmingham, AL, USA) at  $37^{\circ}\text{C}$  for 1 h. Plates were washed 6 times again and developed for 40 min in the dark, using p-nitrophenyl phosphate (Sigma) as a chromagen substrate. The resulting optical density was measured at 405 nm using a UV-max spectrophotometer (Molecular Devices). Negative pre-bleed control sera and a standard mouse anti-CII antiserum were titered on each plate to ensure uniformity of the assay. Antibody binding was expressed as OD<sub>405</sub> units-blank.

**Statistical analysis.** Data were analyzed using the SPSS-PC statistical software (SPSS, Chicago, IL, USA). All comparisons were done between treated and control mice. Group comparisons were conducted using one-way ANOVA. P values  $< 0.05$  were considered statistically significant.

## RESULTS

**Pristane induced arthritis.** Sixteen mice injected with pristane were sacrificed prior to onset of disease (at 1 and 2 months); 28 of the 32 remaining mice injected with pristane developed arthritis. The majority of these mice experienced onset between months 3 and 4, while the remainder had onset between months 4 and 6. Clinical scores observed in the arthritic paws ranged from 1 to 2. Between 4 and 6 months (inclusive), the clinical scores of the mice varied, with scores of 1 predominant in the first month and a rapid progression to joint deformation after 1 month. After the 6 month timepoint, all arthritic mice sacrificed had a score of 2 in 2 or more joints. Each group of 8 mice was then used for proliferation assays, cytokine analysis, and RNA extraction. Control, saline injected mice were subjected to the same protocol at 6 and 12 months. The mice in the PIA group were randomly assigned to a sacrifice date prior to the start of the trial and between 6 and 7 of the 8 mice used at each timepoint had clinically observable arthritis (not including the one and 2 month timepoints prior to onset).

**Proliferation assays.** Spleen and lymph node cells were cultured with a variety of joint components, and at 2 months post-pristane injection, positive proliferative responses above background levels were observed in response to all antigens tested (Figure 1). All joint antigens elicited a positive *in vitro* response at some point after pristane injection, although no proliferative response progressed beyond a low positive level. Elevated responses to antigens preceded the onset of arthritis, which occurred between months 3 and 6. The increase in proliferation was not typically sustained beyond 4 months post-pristane injection, although reactivity to type II collagen was consistently elevated in both the spleen and lymph nodes throughout the study period. Proliferation to HSP was high at one month post-injection, became progressively lower over 4 months, and returned to



**Figure 1.** Proliferative response of spleen cells. Stimulation index (SI) was measured by culturing spleen cells ( $2.5 \times 10^6$  cells/ml) from 8 pristane treated mice with antigen (50  $\mu$ g/ml) and then pulsed with MTT. Data are expressed as mean SI for each timepoint. Figure shows a distinct increase in proliferative response to all antigens tested at 2 months. A unique increased response against HSP was seen at one month. HSP also had the lowest response at 4 months. All antigens show a decreased response at 12 months. In general, the proliferative response diminished by 4 months (average onset time) in most cases, 9 months in all cases. \* $p < 0.05$  for SI of different timepoints.

low levels by 12 months. Fibronectin also had a significantly lower proliferative response at 4 months. These results suggest that the proliferative responses against both connective tissue and systemic antigens peak prior to or around the onset of arthritis and are reduced as the disease progresses.

**Culture cytokine expression.** Cell culture supernatants from antigen bulk cultures were assessed by ELISA for *in vitro* cytokine responses following antigen stimulation (Table 1) to determine whether a predominantly Th1 or Th2 response occurs due to antigen stimulation. Positive cytokine responses (defined as greater than twice the levels detected in medium control) were observed for TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 in response to most antigens; however, IL-4 production *in vitro* remained consistently negative with the exception of mitogen (ConA) stimulation (not shown). IL-6 responses appeared earlier and at higher levels than either TNF- $\alpha$  or IFN- $\gamma$  responses. The strongest IL-6 reactivity occurred

following stimulation with type I collagen, CII, CII 245-270 peptide, pristane joint extract, biglycan, chondroitin sulfate A and B, and aggrecan. All the antigens tested had a positive IL-6 response at one or more timepoints, with all of the antigens tested having a positive IL-6 response at 4 months. This activity may contribute to the onset of arthritis, since disease developed around 4 months post-injection in these animals. This is consistent with previous reports in rats that associate IL-6 with the acute phase of arthritis<sup>35</sup>. TNF- $\alpha$  responses were present in response to all antigens after 4 months, and levels increased over time. Collagen antigens and ConA also stimulated TNF- $\alpha$  in the control mice. At one and 4 months post-pristane injection IFN- $\gamma$  production was seen in response to biglycan, keratin sulfate, and chondroitin sulfate A. At 4 months, an IFN- $\gamma$  response was detected to all the collagens, chondroitin sulfate, and decorin. At either 4 or 12 months, the IFN- $\gamma$  responses were significantly higher than the responses in control (PBS injected) mice to CII peptide, biglycan, decorin, aggrecan, and chondroitin sulfate A. Cultures stimulated with biglycan, decorin, and aggrecan resulted in an increase of TNF- $\alpha$  and IFN- $\gamma$  production, with TNF- $\alpha$  production remaining high throughout the course of disease. This pattern of reactivity over time may contribute to either the joint destruction in arthritis or the failure of joint repair mechanisms, since aggrecan, biglycan, and decorin are major components of cartilage.

**Tissue cytokine levels.** Elevated TNF- $\alpha$  mRNA levels were detected in all spleen tissue samples from one month post-pristane injections (Table 2). IL-1 levels peaked at 4 months post-pristane injection, while TNF- $\alpha$  levels peaked at 6 months. Tissue IL-6 levels were increased in spleens between 2 and 6 months post-injection, with a sharp peak at 4 months post-pristane injection. Elevated IL-4 levels were present in spleen tissue at 4 and 6 months post-pristane injection, while modestly elevated IFN- $\gamma$  levels were seen in spleens from one month to 4 months post-injection. Control (saline injected) mice showed neither positive IL-4 nor IL-6 message in spleen tissue. Arthritic paws showed increased tissue levels of all cytokines tested (Table 2). At 9 months, only the paws showed increased cytokine message. The disparity between the spleen and paw tissue cytokines could be due to the sequestration of autoimmune cells in affected joints in contrast to the activated cells remaining in the spleen. However, it cannot be known for certain that the cytokine changes in the paw are solely due to joint-infiltrating cells, as bone marrow (and to a lesser extent, plasma cells) is not excluded from the nucleic acid extraction.

**Antibodies to connective tissue antigens.** Antibody levels against the antigen library used in culture were investigated by ELISA. Development of autoantibodies against any of the connective tissue or systemic antigens would support the observation that an immune response was occurring. If the autoantibody development corresponded with the prolifera-

**Table 1.** Bulk culture supernatant cytokine levels were measured by ELISA. Data are presented as: +++: > 3× media response, ++: > 2× media response, +: > 1.5× media response, +/-: > 1× media response. 12c: 12 month saline injected control. Pooled cells from 8 mice were cultured with the antigen listed in the Sample column. Biglycan, decorin, and aggrecan resulted in an increase of TNF-α and IFN-γ production. This pattern of reactivity over time may contribute to joint destruction by preventing the formation of new cartilage. Expression of cytokines may vary over time as responses against the various antigens vary over time. A decreased proliferative response may be associated with a decrease in IFN-γ at the 12 month timepoint. CI: collagen type I, CIIP: collagen type II peptide, ChSA/B: chondroitin sulfate A/B, BiP: endoplasmic reticulum chaperone protein, KS: keratin sulfate, PJ: extract from arthritic joints, NJ: normal joints, Ig: aggregated IgG, ConA: concanavalin A, HSP: heat shock protein, GPI: glucose-6-phosphate isomerase, ND: not done.

Sample	TNF				IL-6				IFN			
	1	4	12	12c	1	4	12	12c	1	4	12	12c
CII	–	+	+/-	+/-	–	+	–	–	–	+	–	++
CIIP	–	+/-	++	+/-	–	+++	–	–	–	+++	–	+
CI	–	+/-	++	+/-	+++	+	++	+++	+	+++	–	+++
Biglycan	–	++	+	–	–	+	–	+/-	++	++	–	+
ChSA	–	++	+	–	–	+	–	+/-	–	+	–	+/-
ChSB	–	+	++	–	–	+	+/-	++	–	+/-	–	++
Decorin	–	+	+/-	–	–	+/-	–	+/-	–	+	–	+
BiP	–	+/-	+	–	+++	+	++	+	–	+	+	+++
DNA	–	+/-	++	–	–	+/-	–	–	–	–	–	++
Fibronectin	–	+	++	–	–	+/-	+/-	–	–	–	–	+/-
KS	–	+	ND	ND	++	+/-	ND	ND	+++	+/-	ND	ND
PJ	–	+	+++	ND	–	+/-	++	ND	–	–	+	ND
NJ	–	+	++	ND	–	–	+/-	ND	–	–	+	ND
IG	–	++	++	ND	–	+/-	+/-	ND	–	–	+/-	ND
Aggrecan	–	+	++	–	–	+	+/-	+/-	–	–	++	+
ConA	–	+/-	++	+/-	+++	+++	+++	+++	–	++	+++	+++
HSP	–	+/-	++	–	–	+/-	+/-	+/-	–	–	+/-	+/-
GPI	ND	ND	+	–	ND	ND	–	+/-	ND	ND	–	+/-

**Table 2.** Background cytokine response. Background tissue cytokine was measured by real-time RT-PCR. Data are presented as copy number. The number of samples used is given in the column labeled n. Control spleens were taken at the 6 month timepoint. Bold text indicates mRNA levels higher than PBS injected, nonarthritic controls. Paw cytokine message is consistently increased, indicating a continuing immune response, while spleen message is more variable. IFN-γ is predominant in the spleen one month post-pristane injection and in the paw at 4 months post-pristane injection. This implies a Th1 response is migrating into the paw at the time of onset of arthritis. The immune response could be in the majority of the mice, while the joints remain the targets of autoimmune response. \* Only one spleen had an IL-4 response. ND: not done.

	1 Month	n	2 Month	n	4 Month	n	6 Month	n	9 Month	n	12 Month	n	Control	n
IL-1														
Spleen	9.36 ± 0.45	8	4.82 ± 0.40	6	<b>27.73 ± 1.60</b>	4	11.60 ± 1.22	7	3.64 ± 0.15	6	9.02 ± 4.59	3	16.51 ± 2.43	2
Paw	ND		ND		22.51 ± 2.30	4	ND		24.75 ± 1.64	17	ND		0.00 ± 0.00	15
IL-4														
Spleen	0.00 ± 0.00		<b>339.94 ± 339.94*</b>		<b>15.67 ± 0.73</b>		<b>12.09 ± 2.35</b>		0.00 ± 0.00		<b>4.65 ± 4.65</b>		0.00 ± 0.00	
Paw	ND		ND		11.52 ± 1.29		ND		4.84 ± 2.64		ND		0.00 ± 0.00	
IL-6														
Spleen	0.00 ± 0.00		<b>2.79 ± 1.82</b>		<b>20.39 ± 1.22</b>		<b>3.17 ± 2.08</b>		0.00 ± 0.00		0.00 ± 0.00		0.00 ± 0.00	
Paw	ND		ND		9.05 ± 3.16		ND		5.42 ± 2.94		ND		0.00 ± 0.00	
IFN														
Spleen	<b>17.17 ± 0.85</b>		8.58 ± 1.77		<b>17.04 ± 0.65</b>		9.43 ± 2.66		5.53 ± 1.20		0.00 ± 0.00		13.05 ± 3.21	
Paw	ND		ND		12.24 ± 1.27		ND		13.05 ± 3.21		ND		0.00 ± 0.00	
TNF														
Spleen	<b>14.87 ± 0.81</b>		<b>8.20 ± 2.70</b>		4.87 ± 4.87		<b>12.90 ± 1.23</b>		6.42 ± 0.40		6.42 ± 3.32		7.13 ± 7.13	
Paw	ND		ND		7.58 ± 4.39		ND		23.72 ± 2.02		ND		0.00 ± 0.00	

tive response against an antigen, this might implicate that antigen as important in the pathology of PIA. Antibody production could be instigated by the active CD4+ T cell response against that antigen. The autoantibodies could have multiple effects on the joint: they could prime cells expressing the antigen for complement-mediated lysis, cause the cells to be attacked by macrophages or natural

killer cells, or cause the development of systemic immune complex formation. Positive antibody responses to all connective tissue antigens were present in sera from mice from 2 to 12 months post-pristane injection (Figure 2). Only fibronectin, collagen type II, and joint-extracted antigens exhibited low antibody binding at all timepoints evaluated, while biglycan, chondroitin sulfate B, type I collagen,

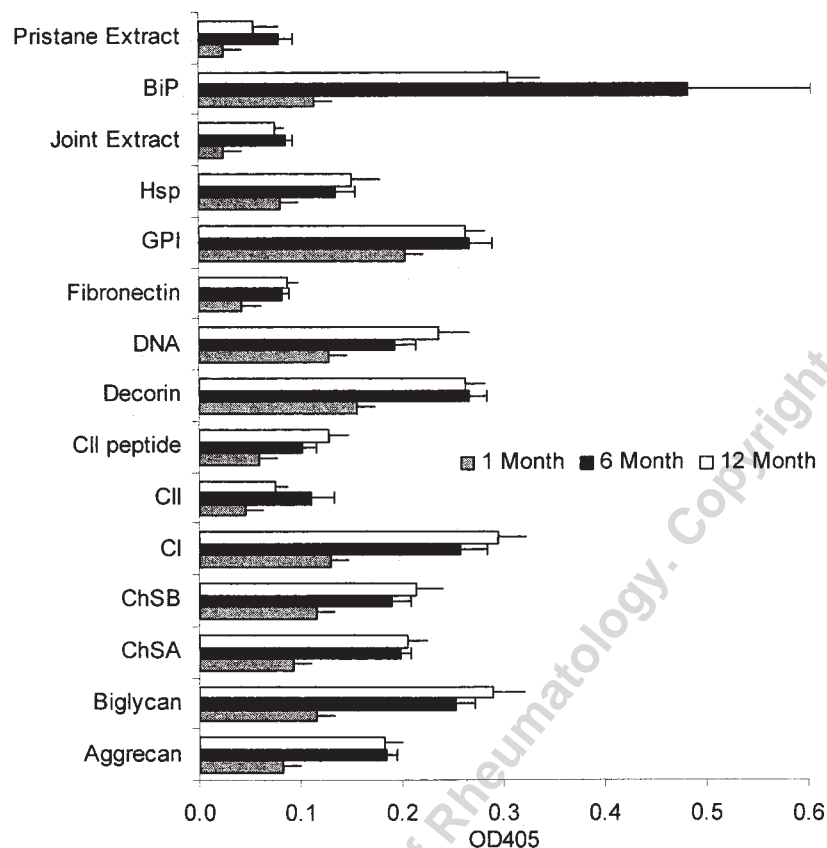


Figure 2. Antibody development following pristane injection. Antibody titers were determined by ELISA. Titers of antibody are shown in OD 405 units. Sera from 8 mice were used at each timepoint. All antibodies were significantly lower at the one month timepoint. All antibody responses were elevated at 6 months post-pristane injection. Antibody titers generally remained elevated through 12 months post-pristane injection.

decorin, DNA, GPI, and biglycan were all positive by one month post-pristane injection, with GPI antibodies reaching their peak levels at one month. Strong antibody binding was observed to biglycan, type I collagen, decorin, DNA, and GPI, and very high reactivity was observed against the chaperone protein BiP at 6 and 12 months. These results indicate that a broad spectrum of antibody reactions precede clinical arthritis, suggesting that the antibody response may play a role in initiation of disease.

## DISCUSSION

Pristane arthritis is induced by a systemic injection of mineral oil, and disease develops between 3 and 6 months after pristane injection. This provides ample opportunity to study the progression of the immune response that culminates in an inflammatory connective tissue disease. PIA presents with erythema and edema in the paw and progresses to joint distortion within 2 months. The histology of PIA is similar to that of RA, with synovitis, marginal erosions, and pannus formation. The time course is quite variable, which is also consistent with the human disease. To determine the development of autoimmunity in PIA, we

examined proliferation, antibody production, and cytokine production in response to a broad array of joint components and systemic antigens. In addition, we assessed the profile of tissue cytokines in arthritic paws and spleens to determine similarities and differences between the elicited responses and immune reactivity at the site of the autoimmune pathological process.

Our results showed that a broad response against the joint occurs in PIA. This is consistent with observations in RA, where there is a response against multiple antigens. The proliferative responses were significantly higher at 2 months post-pristane injection in response to all the antigens tested. Response against HSP was increased at one month and then decreased throughout the remainder of the study. HSP is a relevant antigen in PIA<sup>36</sup> and the proliferative response seen against HSP is significant. However, HSP is not the only potential autoantigen in PIA — the response to CII has also been shown to be relevant in PIA development<sup>37</sup>. The proliferative response against CII or the CII 245-270 immunodominant peptide in our study was not overly impressive, and was similar to the responses to CI, BiP, DNA, aggregated IgG, decorin, fibronectin, aggrecan, normal joint

extract, and pristane joint extract. However, low proliferative responses do not mean that a clinically significant response is not occurring, since CII has consistently exhibited low proliferative responses in collagen induced arthritis, which may be due to the poor solubility of large macromolecules in culture medium. Biglycan, chondroitin sulfate A, and chondroitin sulfate B exhibited a similar pattern of responsiveness as CII, but responses at 2 months were not significantly elevated. Positive proliferative responses against GPI were observed at 9 and 12 months. GPI was only added to the antigen library as its relevance as an arthritis antigen<sup>38</sup> was discovered during the course of our experiments.

We used the same antigen library for bulk culture experiments to determine whether spleen cells would produce similar or different cytokines in response to the broad spectrum of antigens, and whether the profile of cytokine responses would vary with time after pristane injection and the development of disease. Most antigens had positive cytokine responses for TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 at some timepoint during the investigation. The media control cytokine levels in pristane injected mice showed elevation over naive background levels, which implies a generalized activation of the immune system following pristane injection. This finding may be relevant to the observation that pristane injection causes plasmacytoma in some mice, which is accompanied by systemic cytokine expression in the spleen<sup>19</sup>. Upon pristane injection, levels of IL-6 are reported to increase up to 10-fold in peritoneal fluids; in turn, there is an increase in agalactosyl IgG<sup>39</sup>. CI and BiP may be relevant antigens, as they trigger an IL-6 response in culture at one month post-pristane injection, while other antigens did not. IL-4 responses were consistently negative except in response to mitogen stimulation. TNF- $\alpha$  was present in all cultures at 4 months. The proliferative responses were decreasing at 4 months. The continued presence of TNF- $\alpha$  and IFN- $\gamma$  suggests that an immune response is continuing to progress, just not as a proliferative response (i.e., antibody release). Elevated TNF- $\alpha$  production occurs only after 4 months and continues through 12 months. TNF- $\alpha$  has been implicated in the destruction of bone and cartilage as well as being involved in the primary inflammatory response<sup>40,41</sup>. However, the proliferative response does not follow the same trend, implying a change in the focus of the immune response, rather than a change in the presence of the response. It appears that the immune response is focused on joint components in this model. However, a proliferative response is seen against multiple systemic antigens as well. Our findings indicate that an immune response develops prior to joint damage, which would suggest that PIA is an autoimmune, in contrast to a strictly inflammatory, disease. However, there is some evidence of joint damage subsequently provoking autoimmunity, since fibronectin, CII, and BiP antibody responses were increased after development of disease.

Spleen cells, when cultured with medium alone at one and 4 months, produced low levels of TNF- $\alpha$ , IL-6, and IFN- $\gamma$ , suggesting a generalized activation of immune cells. The activation may be due to the systemic administration of pristane that acts as an irritant, as shown by the occurrence of granulomas in the peritoneal cavity and by the appearance of plasmacytomas in mice that do not develop arthritis<sup>19,20</sup>. IL-6 is also seen in rat PIA. Rats that develop chronic arthritis have consistently high levels of IL-6, while in strains in which the arthritis remits, IL-6 levels decrease following the remission of disease<sup>35</sup>. Our results showed a low level IL-6 response concomitant with acute arthritis. RF, anti-DNA, and anti-BiP antibodies are ubiquitously seen in response to pristane injection<sup>20,42,43</sup>. The endoplasmic reticulum chaperone protein BiP is a relevant antigen in RA, where it is overexpressed<sup>44</sup>, and is also a target of T and B cells in collagen induced arthritis<sup>45</sup>. Our experiments indicate that BiP is also a target of B and T cells in PIA.

IL-4 was not elicited directly in response to any antigen stimulation, but was only seen in response to the mitogen ConA. This implies that immune reactivity in PIA is predominantly driven by Th1 mechanisms, which is consistent with the increased IFN- $\gamma$  levels. Increased IL-6 and TNF- $\alpha$  responses imply a chronic inflammatory response to the broad spectrum of antigens. However, there is IL-4 message in the arthritic paws at 4 months, which might indicate the presence of a local attempt at a Th2 response in the joint in an effort to suppress the local Th1 response. A Th2 response in the joint could reduce severity of disease<sup>46</sup> or modulate antibody production. The failure of a regulatory Th2 response may be indicated by the decrease of IL-4 message in the joint by 9 months, when IL-1, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  have increased levels of message in the paw. In RA, IL-4 production *in vitro* was correlated with disease severity, not disease incidence<sup>27</sup>, which might suggest that increased IL-4 could exacerbate disease. Overall, it appears that both Th1 and Th2 mediated responses are required for the occurrence and progression of arthritis.

Tissue cytokine levels were examined to determine whether the systemic immune responses mirrored the activity at the disease site. A Th1 response was observed within the paw, but the spleen showed decreased cytokine production at the 9 and 12 month timepoints, with mRNA levels lower than the control PBS injected mouse spleens. At one month post-pristane injection, IFN- $\gamma$  and TNF- $\alpha$  were the prevalent cytokines. At 2 months, IL-4 and IL-6 message appeared in the spleen, but only one of 6 spleens produced significant IL-4 message. At 4 months post-pristane injection, elevated levels of mRNA for all of the cytokines was seen (TNF- $\alpha$  at low levels). This timepoint corresponds to the onset of arthritis for this group of mice. By 9 months, however, positive mRNA levels for all cytokines were only found in the joints of arthritic mice. This suggests that the systemic response is decreased while the response in the

paw is becoming chronic. The scores of the pristane mice began to increase toward the end of the study. After 9 months, the increase in TNF- $\alpha$  and IFN- $\gamma$  message in the joint may be influencing the rebuilding of cartilage. TNF- $\alpha$  and IFN- $\gamma$  were seen in response to culture with aggrecan, biglycan, and decorin. Since it has been shown that the combined effects of TNF- $\alpha$  and IFN- $\gamma$  decrease the gene expression of these proteins<sup>40</sup>, the observed immunological response may be related to cartilage degradation. In combination with the antibody response seen against aggrecan, biglycan, and decorin, reactivity against critical cartilage components may contribute to the joint destruction in PIA. An immune attack against these proteins may also prevent the repair and rebuilding of the cartilage.

GPI antibodies appear at one month and are present at higher levels than any other antibody tested at that time. The detection of this particular autoantibody may be highly relevant to the immunopathology of arthritis. In the K/BxN mouse, GPI deposits are seen on the surface of the synovial lining cells and on the endothelial cell surface of the arterioles. These deposits are thought to be localized and amplified by IgG and C3 complement<sup>47</sup>. Matsumoto, *et al* proposed that GPI-Ig complexes on articular surfaces initiate inflammation via the alternative complement pathway. This, in turn, would cause extracellular proteins to adhere to cartilage or the joint extracellular matrix, and possibly enhance these proteins as immunological targets. This could explain the comprehensive profile of antibody responses to the joint proteins tested. Joint damage may be initiated by the antibody response to GPI after one month, which may result in the accumulation of GPI-anti-GPI complexes within the joint. An influx of immune cells into the joint and subsequent activation would correspond with the increase in proliferation seen at the 2 month timepoint. These immune cells could amplify the damage to the joint and expose a variety of joint proteins that are normally sequestered, allowing recognition of privileged antigens and subsequent immune reactivity by cells activated by local cytokines within the synovial fluid. This could exacerbate the accumulation of immune complexes containing a variety of antigens within the joint, and lead to a positive feedback cascade culminating in autoimmunity. The release of IFN- $\gamma$  and TNF- $\alpha$  could further prevent the repair of the cartilage damaged in this response, which would result in a cycle of joint destruction culminating in the development of an inflammatory, erosive joint disease.

There is a consistent antibody response to most of the connective tissue antigens tested in DBA/1 mice following pristane injection, and the proliferative response to most antigens is elevated at 2 months post-pristane injection. These findings resemble RA, where patients have a variety of antibody and cellular responses to both joint proteins and systemic proteins. PIA is an appropriate model for addressing the evolution of autoimmunity in RA because

immunological abnormalities arise prior to arthritis development. We recognized that a spectrum of autoimmunity exists in PIA<sup>20,48,49</sup>, which could arise due to (1) one dominant antigen specificity that damages the joint, exposing novel antigens and causing a cascading reaction; or (2) low levels of autoimmunity to connective tissue antigens in susceptible mouse strains could be amplified to pathological significance by pristane stimulation, leading to a broad, joint based autoimmune reaction. Our observation of low levels of reactivity to all the antigens tested lends credence to the latter hypothesis, namely, that the plurality of the reaction is the cause of joint damage in this model as opposed to one particular antigen. The broad specificity of the response seen in PIA is particularly relevant, as it mimics the plurality of the response seen in RA, and hence suggests that broad based autoimmunity might be central to the pathology of RA.

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