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*J Immunol* published online 9 April 2014
http://www.jimmunol.org/content/early/2014/04/08/jimmunol.1303351

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/04/08/jimmunol.1303351.DCSupplemental

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Disease Association and Arthritogenic Potential of Circulating Antibodies against the α1,4-Polygalacturonic Acid Moiety

Hui Dai,* Hong-Liang Dong,† Fang-Yuan Gong,‡ Shu-Liang Sun,† Xiang-Yuan Liu,‡ Zhan-Guo Li,§ Si-Dong Xiong,‡ and Xiao-Ming Gao‡

Much progress has been made in recent years on the diagnostic value, Ag specificity, and pathogenic roles of autoantibodies correlated to the development of rheumatoid arthritis (RA) in humans. However, carbohydrate Ag-specific autoantibodies that may also play important roles in RA have largely been ignored. In this article, we report that serum levels of Abs capable of recognizing α1,4-polygalacturonic acid [(PGA); major structural component of pectin] strongly correlate with RA in humans. The measurements of PGA-specific Abs (PGA-Abs) in sera are comparable to rheumatoid factors and anti–cyclic citrullinated peptide Abs as serological diagnostic markers for RA in terms of sensitivity and specificity. Immunohistochemical staining results indicate that the PGA-Abs selectively bound synovial membrane cells and chondrocytes in the joints of both humans and rabbits (but not rodents). Induction of PGA-Abs by s.c. immunization of rabbits with carrier protein–conjugated synthetic PGA led to severe inflammatory reactions (synovial hyperplasia, small vessel proliferation, and inflammatory cell infiltration) in the joints. Injection of affinity purified anti-PGA IgG into the synovial cavity of rabbits resulted in accumulation of proinflammatory cytokines such as TNF-α, IL-8, and IL-1β in synovial fluid, as well as local pathological damage. We conclude that the PGA–cross-reactive moiety represents a major autoantigen in the joints and can be targeted by autoantibodies capable of triggering arthritogenic responses in vivo. The Journal of Immunology, 2014, 192: 000–000.

Rheumatoid arthritis (RA) is a complex chronic inflammatory autoimmune disease that affects ~0.5% of the adult population worldwide (1, 2). It has long been known to be associated with autoantibodies, most notably rheumatoid factors (RFs) and anti–cyclic citrullinated peptide Abs (CCP-Abs), both of which have been widely used as diagnostic markers for RA since their discovery (3–6). However, association of autoantibodies in RA is likely to extend well beyond RFs and CCP-Abs. Many RA patients have circulating Abs against heterogeneous nuclear ribonucleoprotein A2, type II collagen, or heat shock proteins (7–11). Furthermore, simultaneous assessment of Ab specificities using autoantigen microarrays has revealed multiple reactivities in patient sera (12–14). Monach and colleagues (15) have recently reported that IgG fractions purified from joint immune complexes bound a diverse subset of proteins and peptides from synovium and cartilage. Elevations of a variety of anti-glycan Abs, including IgG, IgA, IgE, and IgM classes, have been demonstrated in a number of inflammatory autoimmune diseases (16–19). However, of studies on the diagnostic value, Ag specificity, and pathogenic potential of autoantibodies correlated to the development of RA in humans, very few touched upon glycan-specific autoantibodies (20–22), even though carbohydrate structures represent a substantial portion of tissue Ags in the joints and could be targeted, just like any other autoantigens, by arthritogenic autoantibodies. Using polysaccharide-based ELISAs, we have previously found positive correlation between RA and serum Abs against dextran and mannann (23, 24). In our more recent screening assays, an even stronger correlation between RA and circulating Abs against pectin and α1,4-polygalacturonic acid [(PGA); major structural component of pectin] was found. This study was designed to investigate 1) diagnostic values of PGA-specific Abs (PGA-Abs) in serum compared with RFs and CCP-Abs; and 2) potential pathogenic effects of PGA-Abs using a rabbit model of PGA-induced arthritis (PIA).

Materials and Methods

Reagents and animals

Polygalacturonic acid (PGA: 81325), pectin (P9135), polygalacturonase (17389), α-1,3-mannan of Saccharomyces cerevisiae (M7504), and β-glucan (89862) were purchased from Sigma-Aldrich (St. Louis, MO). The mAbs against PGA (XA-149) or β-glucan (M1501) were kindly provided by Prof. Boquan Jin (The Fourth Military Medical University, Guangzhou, China). ELISA kits for detection of rabbit IL-8, IL-1β, TNF-α, and IL-10 were from eBioscience (San Diego, CA). ELISA kits for detection of anti-CCP IgG Abs (QUANTA Lite CCP3 IgG ELISA) were from INOVA Diagnostics (San Diego, CA). For quantification of RFs in sera, rate nephelometry was carried out using an IMMAGE Immunochemistry Systems machine (Beckman Coulter, Carlsbad, CA).

Abbreviations used in this article: AS, ankylosing spondylitis; CCP-Ab, anti–cyclic citrullinated peptide Ab; DAB, diaminobenzidine; HS-S, mixed sera from healthy subjects; OA, osteoarthritis; OPD, O-phenylenediamine; PGA, α1,4-polygalacturonic acid; PGA-Ab, PGA-specific Ab; PIA, PGA-induced arthritis; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RA-S, RA sera; RF, rheumatoid factor; ROC curve, receiver operating characteristics curve; XA-149, a PGA-specific mAb.

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Received for publication December 16, 2013. Accepted for publication March 8, 2014.

This work was supported by grants from National Key Basic Research Programs (2010CB529102), the Program for Changjiang Scholars and Innovative Research Team in University (IRT1075), the Natural Science Foundation of China (31370908/31070781/181102253), and the Doctoral Program of Higher Education (20100001120042).

All authors were involved in drafting the article, and all authors approved the final version to be published.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AS, ankylosing spondylitis; CCP-Ab, anti–cyclic citrullinated peptide Ab; DAB, diaminobenzidine; HS-S, mixed sera from healthy subjects; OA, osteoarthritis; OPD, O-phenylenediamine; PGA, α1,4-polygalacturonic acid; PGA-Ab, PGA-specific Ab; PIA, PGA-induced arthritis; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RA-S, RA sera; RF, rheumatoid factor; ROC curve, receiver operating characteristics curve; XA-149, a PGA-specific mAb.

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Female BALB/c (H-2d), C57BL/6 (H-2b) mice and brown Norway rats–10 wk of age were provided by United Bioscience Center, Beijing, China. Female rabbits 8 wk of age were purchased from the Experimental Animal Division of Peking University Health Science Center, Beijing, China. All animals were maintained at the specific pathogen–free laboratory of the Experimental Animal Division of Peking University Health Science Center. All animal experiments were carried out with the permission of Beijing Experimental Animal Management Authority, Beijing, China, at the animal facilities of this department.

Blood samples from healthy human subjects and patients

This study has been reviewed and approved by the Ethics Committee of Peking University Health Science Center. Serum samples were collected from 150 healthy volunteers of both sexes between 20 and 50 y of age. The blood samples were processed within 18 h of collection and the sera stored at –80˚C until use.

Serum samples of 100 RA patients, meeting the American College of Rheumatology 1987 criteria for RA (25) and attending the Department of Rheumatology and Immunology, Peking University People’s Hospital, Beijing, between 2006 and 2009, were collected. At the time of the blood collection, their condition satisfied four of the five following criteria: 1) moderate resting pain; 2) morning stiffness ≥ 1 h; 3) swelling of three or more joints; 4) five or more tender joints; and 5) erythrocyte sedimentation rate (Westergren’s method) ≥ 28 mm/h. The average disease activity score of the patients was 4.2 ± 3.7 (range, 3.2–5.6). Twenty of these patients were in the early phase of their disease (duration, 2–11 mo; average, 7 mo) and thus grouped as “early RA.” Additional information on these patients is given in Supplemental Table I.

For disease-modifying antirheumatic drug treatment, methotrexate (10 mg, once per week), hydroxychloroquine (0.2 g, two times per day), and leflunomide (10 mg, two times per day) were used. For steroid treatment, prednisone (2.5–30 mg, median dose 14.3 mg; oral administration, once per day) or methylprednisolone (100–200 mg, i.v. injection, once per day) was administered.

Serum samples from patients with osteoarthritis (OA, n = 49), ankylosing spondylitis (AS, n = 32), and psoriatic arthritis (PsA, n = 13) who were attending the Department of Rheumatology and Immunology, the Third Affiliated Hospital of Peking University, or People’s Hospital, Beijing, between 2009 and 2012, were also collected as specificity controls (Supplemental Table I).

Purification of PGA-IgG from sera

A PGA column was prepared by conjugating PGA to epoxy-activated Sepharose 6B (Amersham Pharmacia, Piscataway, NJ) following the manufacturer’s instructions; it was then used for affinity purification of PGA-Abs from RA sera (RA-S) or antiserum of rabbits. A sample of sera (20 μl, diluted in 50 ml saline) was passed through the PGA column at a speed of 1 ml/min; bound Abs were washed extensively with PBS, eluted using 0.2 M glycine-HCl (pH 2.7), and neutralized immediately with 1 M Tris-HCl (pH 8.0) before dialysis against PBS. Then PGA-IgG and PGA-IgA (anti-PGA IgG or IgA) were isolated on goat anti-human IgG or IgA conjugated to cyanogen bromide–activated Sepharose 4B (Amersham Pharmacia) and were eluted and dialyzed as above. Rabbit IgG Abs were also purified using a protein G–agarose column (Amersham Pharmacia). The resultant Abs were concentrated with Centricon-10 concentration devices (Millipore, Bedford, MA), quantified by optical absorption at 280 nm on a UV spectrophotometer, and sterile filtered through 0.22-μm filters.

Polysaccharide-based ELISAs

Flat-bottom 96-well microtiter plates (Corning Costar) were coated with 50 μg/ml PGA or pectin in 0.1 M carbonate–bicarbonate buffered saline (pH 9.6) at 4˚C overnight. Each plate was blocked with 1% gelatin (48722; Sigma-Aldrich) in PBS for 2 h at 37˚C. Serum samples were diluted 1:200 in 1% gelatin in PBS and incubated in the ELISA wells for 2 h at 37˚C. To perform inhibition assays, sera were preincubated with 1 mg/ml PGA at room temperature for 1 h before the assay. HRP-labeled detection Abs against IgM, IgG, and IgA of different species were purchased from Southern Biotechnology Associates (Birmingham, AL) and diluted 1:4000 in PBS. The reaction was developed with 100 μl O-phenylenediamine (OPD; Sigma-Aldrich) for 5 min and stopped with 100 μl 2 M H2SO4. OD of the wells was measured at 492 nm, using an ELISA spectrophotometer (Titertek Multiskan Plus MK II; ICN Flow Laboratories, Irvine, U.K.). Affinity purified and lyophilized PGA-specific IgG and IgA from human sera were used as standards for quantitative measurement of specific serum Abs in ELISAs.

Tissue sectioning and staining

Tissue samples from healthy adult animals or human subjects were used for immunohistological screening of PGA moiety expression. Use of autopsied human tissue samples (from otherwise healthy subjects who died of accidents) in this study complied with all human subject guidelines and was approved by Peking University Ethical Committee.

Standard protocol was used to prepare paraffin sections for this study. In brief, human or animal joint, kidney, liver, brain, spleen, lymph node, and thymus were fixed with 4% formaldehyde solution and decalcification, when necessary, followed by paraffin embedding. Then 5- to 8-μm sections were dewaxed and rehydrated through graded alcohol to water immediately before H&E or immunohistochemical staining. For immunohistochemical staining, tissue sections were treated with PGA-specific mAbs for 60 min, followed by HRP-labeled secondary Abs for 30 min. The reaction was performed with 100 μl diaminobenzidine ([DAB]; Sigma-Aldrich) for 5 min and stopped with double distilled H2O. After hematoxylin staining for 30 s and washing completely, sections were dehydrated by passage through a series of alcohol solutions, cleared in xylol, and mounted in MERCKOGLAS (Merck, Whitehouse Station, NJ). The specimens were observed under a light microscope.

Preparation of PGA-BSA conjugates

PGA (15 mg) was incubated with 4 mM sodium periodate in 1.5 ml PBS, pH 7.0, in the dark, at room temperature for 1.5 h. Glycerol was added to consume any residual periodate. The mixture was dialyzed against 0.1 M sodium bicarbonate, pH 8.5, at 4˚C overnight and mixed with 5 mg BSA (Sigma-Aldrich). Sodium borohydride was added to a final concentration of 20 mg/ml, and the mixture was incubated at 37˚C for 4–5 d. Then 0.5 μg sodium borohydride was added to reduce any remaining free aldehyde groups. The conjugate was purified on a Sephacryl S-300 HR 16/60 column (Amersham Pharmacia) and eluted with PBS to separate the conjugate from free BSA. The effect of conjugation was analyzed by NaN300 PAGE (SDS-PAGE). Conjugation was indicated by a progressive increase of a broad high molecular size protein band. Carbohydrate content was determined by the Dubois method (26), and protein concentration was determined using the Coomassie blue protein assay reagent (Pierce, Rockford, IL). The carbohydrate and protein concentration of the resultant PGA-BSA conjugate was estimated to be 1 mg/ml and 3 mg/ml, respectively.

Rabbit model for PIA

Rabbits (three per group) were immunized s.c. with PGA (1 mg), BSA (3 mg), or PGA-BSA (3 mg) emulsified in CFA (Sigma-Aldrich) and boosted twice with the same Ads emulsified in IFA (Sigma-Aldrich) with fortnight intervals. The rabbits were bled from the ear vein every 2 wk to monitor PGA-Ab production. At 2–4 wk after the immunization procedure, the rabbits were sacrificed for blood and hind knee joints. The joints were fixed with 4% formaldehyde solution, followed by decalcification and paraffin section preparation.

In passive transfer experiments, affinity purified rabbit PGA-IgG (0.1 mg/ml in PBS) was injected into the left hind knee joint cavity of recipient rabbits (0.5 ml per rabbit, three per group) on days 0, 3, and 7. As control, purified rabbit IgG at the same dosage was injected into the right hind knee joint cavity of the rabbits. Synovial fluid was harvested from the injected joints of the rabbits immediately before their sacrifice for knee joints on day 14.

Statistical analysis

All experiments were repeated at least three times, and the results are expressed as mean ± SD. Comparison of the groups was performed using the Mann–Whitney U test, whereas differences in the prevalence of the Abs under study were analyzed by Fisher’s exact test in the RA group and controls. Significance was defined as p < 0.05. A receiver operating characteristic (ROC) curve was generated by plotting sensitivity (y axis) against 1-specificity (x axis). Statistical analysis was performed using SPSS software.

Results

Correlation between PGA-Ab prevalence and RA

In our initial screening ELISAs, pectin was recognized by RA-S (an equal proportion mixture of serum samples from 80 RA patients), but not by mixed sera from 150 healthy subjects (HS-S), a finding further confirmed by comparing serially diluted RA-S and HS-S against plate-bound pectin in ELISAs (Supplemental Fig. 1A). Preincubation of RA-S with PGA, the main structural component of pectin (27), almost completely abolished its ability to bind pectin in ELISAs (Supplemental Fig. 1A). After pretreatment (pH,
4.0; 50˚C for 1 h) with pectinase (polygalacturonase), which specifically hydrolyzes the PGA linkage bounds, pectin was no longer recognized by RA-S (Supplemental Fig. 1B), further confirming the linear PGA chain as the major glycotope recognized by RA-S.

To further analyze the correlation between prevalence of PGA-Abs and RA, serum samples from patients with RA (n = 80) or early RA (n = 20), or healthy subjects (n = 150), were individually quantitated for PGA-specific IgA and IgG (PGA-IgA and PGA-IgG, respectively) using PGA-based ELISAs. Average levels of PGA-IgA and PGA-IgG in RA-S and early RA-S were significantly higher than that in HS-S (Mann–Whitney U test, p < 0.0001) (Fig. 1A). ROC curves were plotted to set cutoff values for prevalence assessment and also to evaluate the sensitivity and specificity of the PGA-based ELISAs in RA diagnosis (Fig. 1B). With cutoff values at 4.9 µg/ml (PGA-IgA) and 23.6 µg/ml (PGA-IgG), the prevalence of PGA-IgA and PGA-IgG in RA patients was 73.8% and 80.0%, respectively, significantly higher than that (8.0% and 8.7%, respectively) in healthy controls (2-sided Fisher’s exact test, p < 0.0001). To ascertain the diagnostic specificity of PGA-Abs for RA, serum samples from groups of patients with OA (n = 49), PsA (n = 13), or AS (n = 32) were also analyzed. The results showed that PGA-Abs were absent in sera from patients with OA, PsA, or AS (Fig. 1A), indicating that the correlation between PGA-Abs and RA is not only strong but also highly specific.

Comparison of PGA-Abs with CCP-Abs and RFs as diagnostic markers for RA

Both RFs and CCP-Abs are widely used as serological diagnostic markers for RA in the clinic, with varying specificities and sensitivities (3–6). All 250 serum samples from RA patients and healthy control subjects were sent for RF and CCP-Ab tests in the diagnostic laboratory of The People’s Hospital of Peking University. The sensitivity and specificity of RFs and CCP-Abs in diagnosing RA were calculated and compared with that of PGA-Abs (Table I). When diagnostic specificity was set at 95.3%, PGA-IgA and PGA-IgG ELISAs detected RA with a sensitivity of 65.0% and 77.5%, respectively. Of interest, PGA-IgA detected early RA with a sensitivity of 70%, slightly higher than that of CCP-Abs (66.7%) and much better than RFs (33.3%). When diagnostic sensitivity was set at 80%, PGA-IgA detected early RA with a specificity (93.3%) greater than that of RFs (25.4%) and CCP-Abs (84.7%). These results are in agreement with the ROC curves shown in Fig. 1B. The effectiveness of PGA-IgA in picking up early RA cases is supported by comparison of PGA-IgA, CCP-Ab, and RF levels in sequential serum samples (collected in 2005, 2006, and 2007) from patient PE. The 2005 sample of this patient was positive for PGA-IgA, but negative for CCP-Abs and RFs; he converted to being positive for CCP-Ab and RF a year later (Supplemental Fig. 2).

The PGA glycotope represents a carbohydrate tissue Ag in human joints

An important question with regard to the strong correlation between circulating PGA-Abs and RA is whether the PGA–cross-reacting Ag can be detected in human joint tissues. To address this question, a PGA-specific mouse mAb was prepared using splenocytes from BALB/c mice that had been immunized with carrier protein–conjugated PGA. The resultant mAb—namely,
XA-149—could specifically recognize the linear PGA epitope in ELISAs (Fig. 2A). Given that α1,4-linked galacturono-oligosaccharides of two or three sugars in length were unable to inhibit the binding of XA-149 to pectin or PGA (Supplemental Fig. 1C), the minimum size of the XA-149–specific glycotope was estimated to be equal to or larger than four sugars in length. Next XA-149 was used in immunohistochemical analysis on paraffin sections of human tissues, including the spleen, lung, kidney, liver, lymph node, brain, articular cartilage, and joint synovium. Of note, synovial membrane and chondrocytes in the cartilage, but not any other tissues examined therein, were positively stained (Fig. 2B). Isotype control Abs, as well as HRP-labeled Abs alone, were included in parallel staining experiments with negative results (data not shown). These data support the idea that the PGA structure-containing glycosylation moieties constitute carbohydrate autoantigens in the joint tissues of humans.

A rabbit model for PIA

Rodents would be ideal model animals for investigating potential pathogenic roles played by PGA-Abs in vivo. Unlike humans, however, naive mice and rats possess high titer circulating natural Abs (IgM, but not IgG or IgA) against pectin and PGA (data not shown). Of interest, rabbits came up as a relatively clean “natural PGA-Ab–negative” species, and immunohistochemical staining with XA-149 against rabbit tissue sections revealed a PGA moiety tissue expression profile similar to that of humans, that is, positive expression by synovial membranes and chondrocytes in articular cartilage, but not by any other tissues examined (Fig. 3).

### Table I. Diagnostic sensitivity and specificity of different serum Abs for RA and early RA

<table>
<thead>
<tr>
<th>Patient Group/Cutoff Value</th>
<th>PGA-IgA</th>
<th>PGA-IgG</th>
<th>CCP-Abs</th>
<th>RFs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity 95.3%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cutoff 6.9 µg/ml</td>
<td>28.1 µg/ml</td>
<td>29.5 U/ml</td>
<td>160 U/ml</td>
</tr>
<tr>
<td>Early RA</td>
<td>70.0%</td>
<td>25.0%</td>
<td>66.7%</td>
<td>33.3%</td>
</tr>
<tr>
<td>RA</td>
<td>65.0%</td>
<td>77.5%</td>
<td>76.9%</td>
<td>76.7%</td>
</tr>
<tr>
<td>Specificity 99.3%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cutoff 7.8 µg/ml</td>
<td>36.0 µg/ml</td>
<td>87.5 U/ml</td>
<td>215 U/ml</td>
</tr>
<tr>
<td>Early RA</td>
<td>65.0%</td>
<td>20.0%</td>
<td>54.1%</td>
<td>30%</td>
</tr>
<tr>
<td>RA</td>
<td>57.5%</td>
<td>67.5%</td>
<td>65.4%</td>
<td>73.7%</td>
</tr>
<tr>
<td>Sensitivity 80.0%&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Early RA</td>
<td>93.3%</td>
<td>57.3%</td>
<td>84.7%</td>
</tr>
<tr>
<td>RA</td>
<td>88.0%</td>
<td>91.3%</td>
<td>85.3%</td>
<td>81.5%</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ra diagnostic sensitivity of PGA-IgA, PGA-IgG, CCP-Abs, and RF are compared, setting the diagnostic specificity at 95.3% or 99.3%. Cutoff values of the assays are also shown.

<sup>b</sup>When RA diagnostic sensitivity was set at 80%, corresponding specificity values of the assays for PGA-IgA, PGA-IgG, CCP-Abs, and RFs are compared.

**FIGURE 2.** Specificity of mAb XA-149 and detection of PGA–cross-reacting Ag in human tissues. (A) The mAbs XA-149 and M1501 were serially diluted and assayed in ELISAs using polyvinyl plates precoated with PGA, β-glucan, or α1,3-mannan. The detection substrate was OPD and the results were expressed as mean absorbance at 492 nm ± SD. (B) For immunohistochemical staining, paraffin tissue sections of human spleen, lung, kidney, liver, lymph node, brain, articular cartilage, and knee joint synovium were treated sequentially with XA-149 (10 µg/ml) and HRP-labeled secondary Ab (1/1000 dilution), followed by DAB as substrate. The stained specimens were observed under a Leica epifluorescence microscope, at original magnification ×100, and the results recorded using a charge-coupled device system. Scale bar, 50 µm. Preincubation of mAb XA-149 with soluble PGA destroyed its ability to stain human synovial membrane cells and chondrocytes, thereby confirming the specificity of the staining results (B). Arrows indicate positively stained cells.
Rabbits were used in subsequent in vivo studies on the arthritogenic potential of PGA-Abs, in which female rabbits (three per group) were s.c. immunized with either BSA-PGA, or BSA, or PGA, in CFA and boosted twice with the same Ags in IFA on weeks 2 and 4. High-titer serum PGA-IgG was detectable, by ELISAs (dilution endpoint 1/800–1/1600), in PGA-BSA–vaccinated rabbits 2 wk after the third immunization (Fig. 4A), which remained at high levels for at least 4 wk thereafter. PGA-IgA was also present in sera from rabbits immunized with PGA-BSA, albeit at lower titers than were found with IgG (Fig. 4A). Only low-level anti-PGA serum IgM Abs (dilution endpoint 1/100–1/200) were detected in the PGA-BSA and PGA groups. Immunization with BSA or PGA alone did not induce PGA-specific IgA or IgG in rabbits. At 5–6 wk after the immunization procedure, the rabbits were sacrificed for their hind knee joints, for histochemical examination. Microscopic observation of the H&E-stained tissue

![PGA moiety expression profile in rabbit](image)

FIGURE 3. PGA–cross-reactive Ag in rabbit tissues. Paraffin tissue sections of rabbit heart, liver, spleen, lung, kidney, lymph node, articular cartilage, and hind knee joint synovium were treated sequentially with XA-149 (10 μg/ml) and HRP-labeled secondary Ab (1/1000 diluted), followed by DAB as substrate. As controls, anti-PGA Abs were incubated with PGA and then used as the first Ab staining specimens of cartilage and synovium. The stained specimens were observed under a Leica epifluorescence microscope, at original magnification ×100, and the results recorded using a charge-coupled device system. Scale bar, 50 μm.

![PGA moiety expression profile in rabbit](image)

FIGURE 4. Knee joint inflammation following induction of PGA-Abs in rabbits. (A) The 2-mo-old female white rabbits (three per group) were s.c. injected with PGA, BSA, or PGA-BSA and boosted 2 wk and 4 wk later. The mice were bled in week 6, and the sera were assayed for PGA-Abs. The detection Ab was HRP-conjugated goat anti-rabbit IgG, IgA, and IgM with OPD as substrate, and the results were expressed as OD 492 nm with SD. Hind knee joints from rabbits immunized with PGA-BSA (B), or PGA, or BSA, or unimmunized (C) were fixed with 4% formaldehyde solution and then decalcified, followed by paraffin section preparation and H&E staining. Microscopic observation at original magnification ×100 revealed obvious inflammatory changes, including synovial hyperplasia, small vessel proliferation, and inflammatory cell infiltration, in the joint sections from rabbits immunized with PGA-BSA, but not PGA or BSA.
sections revealed obvious inflammatory changes, including synovial thickening, papillary hyperplasia, small vessel proliferation, and inflammatory cell (mostly polymorph neutrophils and lymphocytes) infiltration, in the joints of the PGA-BSA group (Fig. 4B). No similar histological changes were evident in the PGA-immunized, BSA-immunized, or unimmunized groups (Fig. 4C). The PIA in rabbits appeared to be self-limiting. Of the rabbits that had been maintained for 12 wk after the third immunization with BSA-PGA, no severe joint swelling or movement difficulties were observed, although a longer period of observation and more thorough histological examination of the joints at different time points after the immunization procedure will be required for a more definitive conclusion.

**Evidence for pathogenic potential of PGA-Abs in vivo**

To further assess whether the PGA-Abs could cause arthritic damage in vivo, PGA-IgG was affinity purified from PGA-specific rabbit antiserum and then injected into the left hind knee joint cavity of naive rabbits (three doses in 7 d, 50 μg/day per joint, three rabbits per group), with PGA–nonspecific rabbit IgG injected into the right hind knee joint as control. The recipient rabbits were sacrificed 2 wk later for their knee joints, for histological examination. As shown in Fig. 5A, the ELISA results confirming PGA specificity of the affinity purified Abs. Concentrations of IL-8, IL-1β, TNF-α, and IL-10 in the synovial fluid of the rabbits, collected immediately before their sacrifice, were determined using ELISA kits. As shown in Fig. 5B, IL-8, IL-1β, and TNF-α, but not IL-10, were significantly elevated in the synovial fluid of the PGA-IgG–treated group, compared with the control. Consistent with cytokine detection findings, strong inflammatory reactions were also observed in the joint tissues of the PGA-IgG–treated rabbits, but not in the control group (Figs. 5C, 5D).

**Discussion**

Our present work illustrates strong correlation between circulating PGA-Abs and the development of RA, but not OA, PsA or AS, in humans. Sample sizes in this study are relatively small, but the data presented in this article nevertheless indicate the potential usefulness of PGA-Abs, particularly PGA-IgA, as serological diagnostic markers for RA. We propose that the combination of PGA-Abs with CCP-Abs and RFs is likely to improve substantially the specificity and sensitivity of RA diagnosis in clinic.

Another important finding of this study is almost exclusive expression of the PGA–cross-reacting Ag (recognizable by mAb XA-149) in the joints of humans and rabbits. Immunohistochemical staining of joint tissue sections from mice and rats using XA-149 produced negative results (data not shown). The PGA structure has not previously been described in animals, and information on enzyme(s) responsible for synthesizing the PGA epitope–containing carbohydrate moiety in mammalian cells is also lacking. However, our results are supported by earlier reports of Gilboa-Garber and coworkers (28) that the polygalacturonic acid–specific gonad lectin of *Aplysia depilans* was able to bind erythrocytes from mice, rabbits, and humans independent of ABO blood groups. In addition, Fang and colleagues (29) documented a new *Phaseolus vulgaris* lectin (namely, BTKL) highly specific for PGA. BTKL was able to exert selective toxicity on human liver carcinoma Hep G2 cells and also act upon murine splenocytes and induce production of various cytokines by the cells (29). Nevertheless, the possibility that mAb XA-149 cross-reacts with a chemical entity of mammalian cells that structurally mimicks the PGA moiety cannot be excluded at the present time. Ongoing projects in this laboratory will analyze the carbohydrate structures on the glycoproteins of synovial membrane cells, using mass spectrometry.

Several studies of rheumatoid joints have suggested that Abs mediate disease locally in autoimmune arthritis. It is evident that complement activation occurs locally in inflamed joints of RA (30). IgG and complement C3 are found together on the cartilage surface, in the synovium and within phagocytes (15, 31). Animal experiments have confirmed that Abs to various joint components (e.g., type II collagen) are sufficient to elicit destructive joint inflammation (32–36). In our study, induction of PGA-Abs by s.c. immunization in rabbits led to severe arthropathies; direct injection of affinity purified PGA-IgG into the synovium cavity also caused proinflammatory cytokine production and local inflammation (Figs. 4 and 5). Affinity purified PGA-IgG from sera of RA patients or BSA-PGA–immunized rabbits was able to positively stain synovial membrane and cartilage chondrocytes in joint tissue sections (data not shown), thereby sustaining the hypothesis that the arthritogenicity of anti-PCA (auto)antibodies is due to their interaction with joint-expressed antigenic targets. No obvious destructive damage to cartilage and bone in the joint was observed in our PIA rabbit model. Given that RA is a slowly progressing disease, however, a much longer observation period may be necessary for a definitive conclusion.

It is also of importance to compare PGA-Abs with RFs and CCP-Abs for their pathogenic roles in joint inflammation in RA. The majority of RA patients possess RFs; however, patients with other chronic inflammatory or infectious diseases also produce RFs (3, 4, 6, 8), implying that the mere presence of RFs is insufficient for a chronic joint inflammatory response. CCP-Abs are much more...
specific for RA, and citrullinated proteins are found in the inflamed joint (5, 6, 37). CCP-AbS have been shown to enhance tissue damage in experimental autoimmune arthritis (38), but direct evidence for a pathogenic role in inflammatory damage in RA is still lacking. Of interest, PGA-AbS seem to fulfill all three criteria of Witebsky’s postulate of autoimmunity (39): They are predictive for RA; PGA–cross-reactive epitopes are specifically expressed in the joint; and induction of PGA-specific humoral response, or intrasynovial injection of PGA-IgG, led to joint inflammation in animal models. These results point toward an important role for PGA-AbS in the pathogenesis of RA. A possible mechanism by which PGA-AbS exert arthritisogenic effects in vivo is directly inducing proinflammatory cytokine production by synovial cells and/or innate immune cells in local tissues. In our FACS analysis, however, mAb XA-149 and affinity purified PGA-AbS failed to stain viable peripheral blood monocytes from healthy human subjects and synovial fibroblasts derived from rabbits (not shown). Future studies in this laboratory will aim to characterize in further detail the expression pattern of PGA epitope-containing Ags in synovial cells in relation to their proinflammatory cytokine secretion and biological function.

With the data taken together, several conclusions may be drawn from this study: 1) Circulating PGA-Ab measurements strongly correlate with RA and may be valuable in assisting RA diagnosis in the clinic; 2) the PGA–cross-reacting moiety is specifically expressed in human joint tissues and could be a target for arthritisogenic autoantibodies; 3) tissue distribution of the PGA epitope-containing moiety in rabbits is similar to that in humans, and induction of PGA-AbS in rabbits results in arthritis damage; and 4) directly inoculated PGA-AbS cause local inflammatory damage to rabbit joints, providing direct evidence for the pathogenic potential of PGA-AbS. These results may have important implications for our understanding of the role of Abs against carbohydrate autoantigens in the development of RA. If PGA-AbS truly are direct inducing proinflammatory cytokine secretion and biological function.

Acknowledgments
We thank Prof. B.Q. Jin (Fourth Military Medical University of People’s Liberation Army, Guangzhou, China) for preparation of mAbs XA-149 and M1501 and Profes. F.Y. Liew (University of Glasgow, Glasgow, U.K.) and P. Delves (University College of London, London, U.K.), for useful comments on the manuscript.

Disclosures
The authors have no financial conflicts of interest.

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