Association between anti-\(\alpha\)-1,4-D-polygalacturonic acid antibodies and Henoch-Schönlein purpura in children

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Abstract

**Objective:** To investigate the relationship between anti-\(\alpha\)-1,4-D-polygalacturonic acid (PGA) antibodies, particularly immunoglobulin (Ig)A, and Henoch-Schönlein purpura (HSP) in children.

**Methods:** This observational case–control study investigated PGA-IgA, PGA-IgG, and PGA/PGA-IgA circulating immune complex (PGA/PGA-IgA CIC) in paediatric patients with HSP versus controls. Children with HSP were also evaluated for food specific IgG and food intolerance. Between-group differences in anti-PGA antibodies were analysed.

**Results:** Serum PGA-IgA and PGA-IgG levels were significantly increased in patients with acute HSP (\(n = 251\)) versus those with urticaria (\(n = 48\)), acute respiratory infections (\(n = 95\)), surgical controls (\(n = 53\)) and neonates (\(n = 92\)). PGA/PGA-IgA CIC levels were also significantly higher in the acute HSP group versus surgical control and neonate groups. Levels of PGA/PGA-IgA CIC and PGA-IgA were significantly correlated (\(r = 0.997\)), and PGA-IgA showed high diagnostic specificity for HSP. No statistically significant differences were observed in PGA-IgA and PGA-IgG between various degrees of food intolerance in children with HSP.

**Conclusion:** Increased anti-PGA antibodies, particularly PGA-IgA and PGA/PGA-IgA CIC, were significantly associated with acute HSP in children. Food intolerance was not found to be associated with increased anti-PGA antibodies in children with HSP.

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**Introduction**

Henoch-Schönlein purpura (HSP), also known as immunoglobulin (Ig) A vasculitis (IgAV), is a systemic disease involving the capillaries and is characterized by leukocytoclastic vasculitis. More commonly seen in childhood, HSP often occurs among both preschool and school-age children, mainly affecting the skin, gastrointestinal tract, joints and kidneys. In clinical settings, the main disease manifestations include arthritis, non-thrombocytopenic purpura, abdominal pain and kidney disorders.

The pathogenesis of HSP remains unclear, but is generally considered to be a comprehensive and complicated process. Due to the enhancement of auxiliary T and B lymphocyte activity, a large number of IgA circulating immune complexes (CICs) are produced, that accumulate in the small vascular walls of affected organs and tissues. Small-vessel vasculitis may be induced through the complement-mediated immune inflammatory reaction, and IgA is thought to play a key role in HSP pathogenesis. CICs associated with non-glomerulonephritis-type HSP usually exhibit a small molecular weight, while CICs associated with glomerulonephritis exhibit a high molecular weight. The IgA type anti-endothelial cell antibodies, anti-cardiolipin antibodies, anti-neutrophil cytoplasmic antibodies, anti-rheumatoid factor antibodies, and anti-lysosomal-associated membrane protein 2 antibodies have all been reported to display elevated levels in the serum of patients with HSP. These specific IgA antibodies may play an important role in the pathogenesis of non-glomerulonephritis type HSP by forming small molecule CICs that deposit on the wall of small vessels through binding with antigen or circulating antigen.

Antibodies against pectin and its main component, α-1,4-D-polygalacturonic acid (PGA), have been found in patients with rheumatoid arthritis (RA), and are reported to be related to RA pathogenesis. Pectin is mainly composed of polygalacturonic acids, and can form gels and is partially methoxylated under suitable conditions. Pectin has a strong binding force with water due to its hydrophilic groups, and is widely used in the food, pharmaceutical and cosmetics industries. The pathogenesis of HSP has been associated with the use of certain food, drugs and cosmetics, but there is currently no published research on whether HSP pathogenesis may be related to pectin-based additives. There are no PGA structural components or PGA cross-reaction antigens in the human spleen, lung, kidney, liver, lymph nodes and brain, which suggests that there is no PGA structural component or PGA cross-reaction antigen on the surface of human vascular intima. CICs comprising food antigen and antibody could mediate the immune inflammatory reaction. Thus, the PGA antigen from food sources and the anti-PGA antibody, particularly an increase in CICs produced by the binding of PGA antigen to IgA, may play an important role in HSP pathogenesis, particularly the non-glomerulonephritis-type HSP.

The aim of the present study was to analyse serum levels of anti-PGA IgA.
(PGA-IgA), anti-PGA IgG (PGA-IgG), and PGA/PGA-IgA CICs in children with acute HSP without renal damage versus various control groups. In addition, the association between food intolerance and PGA antibodies in patients with HSP were investigated.

Patients and methods

Study population

This observational case–control study included paediatric patients at the Children’s Hospital of Soochow University, who were diagnosed with HSP according to the 2006 paediatric vasculitis classification criteria of the European League Against Rheumatism (EULAR) and the Paediatric Rheumatology European Society (PRES), between April 2015 and October 2016. Inclusion criteria were as follows: no renal damage; no microscopic haematuria; and normal levels of immunoglobulin G, α1 microglobulin, β2 microglobulin, urinary transferrin, N-acetyl glucosaminidase, and urine microalbumin. Exclusion criteria comprised recurrent HSP, immunological diseases, prior or current treatment with antihistamines, adrenal cortical hormone, or immunosuppressive therapy. Upper respiratory tract infection is a common predisposing factor for HSP in children, and mucosal immunity affects the activity of IgA. For study comparisons, paediatric patients with acute urticaria and/or acute respiratory infection were included as case controls; paediatric patients with hernia, circumcision or polydactyly who received surgery were included as normal controls; and neonates were included for background serum antibody levels.

The study protocols were approved by the Ethics Committee of the Children’s Hospital of Soochow University, and written informed consent was provided by the parents/legal proxies of each study participant.

Sample collection

Peripheral venous blood samples (3–5 ml) were collected from each participant. Samples were placed at room temperature and serum was separated within 4 h of sample collection. Serum samples that were not analysed within 8 h of collection were stored at –80°C prior to analysis, and repeated freeze-thaw cycles were avoided.

Serum PGA-IgA and PGA-IgG determination

Briefly, 50 μg/ml PGA (Sigma-Aldrich, St. Louis, MO, USA) was added to Nunc™ 96-well plates (Thermo Fisher Scientific, Roskilde, Denmark) and incubated at 4°C overnight. The plates were then blocked by incubating with 3% bovine serum albumin (BSA; Solarbio, Beijing, China) at 4°C overnight. Serum samples were diluted with 3% BSA (1:200 dilution) and 100 μl of each sample was added to PGA-coated wells of the 96-well plate, and incubated at 37°C for 2 h. Plates were then washed three times with 0.05% Tween-20, and 100 μl of horse-radish peroxidase (HRP)-conjugated IgA or HRP-conjugated IgG (Santa Cruz, CA, USA; both diluted 1:4 000 with phosphate buffered saline [PBS; pH 7.4]) was added to each well. Plates were covered and incubated at 37°C for 1 h before washing four times with 0.05% Tween-20. The signal was developed for 30 min at 37°C using a chromogenic reagent kit (Yinke Xinchuang Technology, Nanjing, China) prior to adding stop solution (Yinke Xinchuang Technology), according to the manufacturer’s instructions. The signal was evaluated using a microplate reader (Bio-Rad, Hercules, CA, USA) to obtain optical density (OD) values at 450 nm/630 nm.
**Immunogen preparation and serum PGA/PGA-IgA CIC determination**

The immunogen was prepared by a glutaraldehyde cross-linking method. Briefly, 8 mg of keyhole limpet hemocyanin (an immunogenic carrier protein) was mixed with excessive glutaraldehyde for 10 min to allow conjugation. Residual glutaraldehyde was removed, then 6 mg of PGA was added and the mixture was incubated for 3 h at room temperature. For the immunization, two New-Zealand rabbits (aged 4–6 weeks; weight, 2–3 kg) were challenged with the immunogen preparation in Freund’s adjuvant at a 500-µg dose delivered via subcutaneous injection, once every 2 weeks. The rabbits were housed using a 12-h light/12-h dark cycle with free access to food and water, and the study protocols followed the Jiangsu Provincial Laboratory Animal Management Regulations to ensure animal welfare. Pre-immune serum was collected and used as a blank control to evaluate serum titre during antibody production. Immune serum samples were collected and diluted with PBS (pH 7.4), followed by washing the antibody with glycine solution (pH 3.0) and neutralizing with Tris-HCl (pH 8.3) until a pH value of 7.2–7.4 was achieved. The anti-PGA polyclonal antibodies were purified by immunoaffinity chromatography.

Following antibody preparation, the study samples were analysed for PGA/PGA-IgA CIC using enzyme-linked immunosorbent assay (ELISA). Briefly, Nunc™ 96-well plates (Thermo Fisher Scientific) were coated with 120 µl/well of the prepared rabbit anti-PGA polyclonal antibody (1:3000 dilution) and incubated overnight at 4°C. Serum samples and HRP-conjugated secondary antibodies were added, incubated and washed as described above. The developed signals were evaluated using a microplate reader (Bio-Rad, Hercules) to obtain OD values at 450 nm/630 nm, as described above.

**Food intolerance**

The presence of food-specific IgG in serum samples was determined using a commercial ELISA kit (Food Intolerance 14G ELISA, Cat No. 7194; Biomerica, Irvine, CA, USA) according to the manufacturer’s instructions. The presence of IgG was determined for the following 14 foods: beef, chicken, morrhua, corn, crab, albumen/egg yolk, mushroom, milk, pork, rice, shrimp, bean, tomato and wheat; and the IgG concentration was determined using OD values at 450 nm/630 nm, as described above. The results were graded as follows: 0 (<50 U/ml), negative; +1 (50–100 U/ml), slightly sensitive; +2 (101–200 U/ml), moderately sensitive; +3 (>200 U/ml), severely sensitive.

**Statistical analyses**

Data analyses were performed using SPSS software, version 22.0 (IBM, Armonk, NY, USA). Continuous (measurement) data that were not normally distributed are presented as median (interquartile range) and normally distributed continuous data are presented as mean ± SD. Between-group comparisons were performed using independent samples Kruskal–Wallis one-way analysis of variance (ANOVA). Spearman’s rank correlation coefficient was used to analyse the correlation between measurement data that were not normally distributed. The sensitivity and specificity of anti-PGA-IgG and anti-PGA-IgA in detecting patients with HSP was assessed using receiver operating characteristic (ROC) curves. A P value <0.05 was considered to be statistically significant.

**Results**

A total of 251 patients with HSP (139 male: 112 female patients; mean ± SD age, 6.61
± 5.14 years; range, 1–15 years) were included in this study. Other study groups included 48 patients with acute urticaria (28 male: 20 female patients; mean age, 5.34 ± 6.94 years; range, 1.5–13 years), 95 patients with acute respiratory infection (47 male: 48 female patients; mean age, 4.99 ± 5.66 years; range, 2–14 years), 92 neonates (48 male: 44 female patients; mean age, 7.51 ± 13.84 days; range, 1–29 days), 53 patients who received surgery for either hernia, circumcision or polydactyly (28 male: 25 female patients; mean age, 4.55 ± 3.16 years; range, 1–13 years).

**Serum anti-PGA antibody and PGA/PGA-IgA CIC were increased in children with HSP**

Statistically significant differences in the distribution of serum PGA-IgA (F = 318.9, P < 0.001), PGA-IgG (F = 81.8, P < 0.001) and PGA/PGA-IgA CIC (F = 172.1, P < 0.001) were shown between the different study groups (Table 1; Independent samples Kruskal–Wallis one-way ANOVA). In the HSP group, PGA-IgA levels were significantly increased compared with the other four groups (P < 0.001), while levels in the neonate group were significantly lower than the other groups (P < 0.001). PGA-IgA levels in the acute respiratory tract infection and urticaria groups were significant increased compared with the surgical control group (P < 0.05, Figure 1a; Independent samples Kruskal–Wallis one-way ANOVA). No statistically significant differences in PGA-IgG levels were observed between the acute respiratory infection, urticaria and surgical control groups (P > 0.05), however, PGA-IgG levels in these groups were significantly decreased compared with the HSP group (all P < 0.05), and were significantly higher compared with the neonate group (P < 0.05, Figure 1b; Independent samples Kruskal–Wallis one-way ANOVA). Levels of PGA/PGA-IgA CIC in patients with HSP were significantly increased compared with surgical controls (P < 0.001), and PGA/PGA-IgA CIC levels in the surgical controls were significantly increased versus the neonate group (P < 0.001; Figure 1c; Independent samples Kruskal–Wallis one-way ANOVA). Serum PGA-IgA levels were found to be significantly correlated with PGA/PGA-IgA CIC levels (r = 0.997, P < 0.001; Figure 1d) in patients with HSP.

| Study group                     | Patient n | PGA-IgA    | PGA-IgG    | PGA/PGA-IgA CIC |
|--------------------------------|-----------|------------|------------|-----------------|
| HSP                            | 251       | 1.48 (1.03–1.77) | 2.35 (1.89–2.86) | 1.13 (0.79–1.72) |
| Urticaria                      | 48        | 0.77 (0.42–1.13) | 2.09 (1.84–2.29) | NA              |
| Acute respiratory infection    | 95        | 0.73 (0.52–0.87) | 2.09 (1.70–2.32) | NA              |
| Surgical control               | 53        | 0.53 (0.35–0.68) | 2.14 (1.86–2.28) | 0.61 (0.31–0.95) |
| Neonates                       | 92        | 0.24 (0.10–0.44) | 1.54 (1.46–2.09) | 0.03 (0–0.10)   |
| Statistical significance       |           | P < 0.001   | P < 0.001   | P < 0.001       |

Data presented as median (interquartile range). PGA, α-1,4-D-polygalacturonic acid; Ig, immunoglobulin; CIC, circulating immune complexes. P < 0.001, statistically significant between-group differences (independent samples Kruskal–Wallis one-way analysis of variance).
Roles of PGA-IgA and PGA-IgG in diagnosing HSP in children

ROC curves were used to investigate the roles of PGA-IgA and PGA-IgG in diagnosing HSP. The area under the curve for PGA-IgA and PGA-IgG was 0.91 and 0.74, respectively ($P < 0.001$). A cut-off value of 0.82 for PGA-IgA was associated with a sensitivity of 90.9% and specificity of 81.0%. At a cut-off value of 2.28 for PGA-IgG, the sensitivity was 64.5% and the specificity was 78.8%. These results suggest that PGA-IgA showed high diagnostic value for HSP and was highly specific (Figure 2a), and PGA-IgG showed moderate diagnostic value (Figure 2b).

Figure 1. Serum PGA-IgA, PGA-IgG and PGA/PGA-IgA CIC levels between paediatric patients with Henoch-Schönlein purpura (HSP), neonates, surgical controls, and patients with acute respiratory infection or with urticaria: (a) between-group differences in serum PGA-IgA levels; (b) between-group differences in serum PGA-IgG levels; (c) between-group differences in serum PGA/PGA-IgA CICs; and (d) correlation between PGA-IgA and PGA/PGA-IgA CIC levels in patients with HSP. Data presented as median and interquartile range; $P < 0.05$, statistically significant between-group differences (Kruskal–Wallis one-way analysis of variance). PGA, α-1,4-D-polygalacturonic acid; Ig, immunoglobulin; CIC, circulating immune complexes; OD, optical density at 450/630 nm.
Food intolerance was not associated with increased anti-PGA antibody in children with HSP

Among 251 paediatric patients with HSP, 236 (94.02%) showed some level of food intolerance (graded +1, +2 or +3). The distribution of serum PGA-IgA and PGA-IgG levels was not found to be significantly different between various degrees of food intolerance in these patients \((P > 0.05\); Figure 3).

Discussion

Pectin is commonly used in the food, pharmaceutical and cosmetic industries, and PGA is its major component. A study that investigated the correlation between PGA and RA found that PGA-IgG and IgA levels are significantly increased in patients with RA, and circulating anti-PGA antibody was shown to be closely related to the pathogenesis of RA. The present data showed that anti-PGA antibodies...
and PGA/PGA-IgA CICs were significantly increased in patients with acute HSP without renal injuries versus all other groups. Despite the fact that levels of PGA-IgG were higher than PGA-IgA levels, the elevation of PGA-IgA versus other groups was more pronounced than that of PGA-IgG, and the diagnostic specificity of PGA-IgA was superior to that of PGA-IgG.

In the serum of neonates, PGA antigen can be used for the recognition of natural IgM antibody. The natural antibody spectrum is known to be affected by genetic and environmental factors. The PGA in microorganisms, food or breast milk, or antigenic elements containing the PGA structural domain, or autoantigens may be the source of generated antibodies. In individuals with HSP susceptible to PGA or PGA epitopes, there is a possibility that immune memory cells may be present. These patients may be in a state of immune tolerance after in vivo regulation. In cases of immune imbalance, there might be aberrant activation of T and B lymphocytes, which may then trigger the aberrant expression of anti-PGA antibodies.

To date, the presence of PGA component and/or PGA cross reacting antigen on the surface of the vascular intima remains unclear. Elevated levels of PGA-IgA or PGA/PGA-IgA CIC, together with their accumulation in the small vascular walls, may be associated with the pathogenesis of HSP. IgA is well known to present in the form of a monomer or polymer. For example, in the mucosal tissues, IgA has been shown to mainly present as a polymer, while in the peripheral circulation, IgA takes the form of a monomer. Interestingly, IgA was mainly found in the form of a polymer in patients with IgA nephropathy. PGA, commonly found in food, drugs and cosmetics, may trigger food intolerance in children with HSP, and the generation of PGA-IgA by mucosal immunity in the presence of PGA-containing food may be a cause for elevated PGA/PGA-IgA CICs. The present data indicated that food intolerance was not associated with elevated levels of PGA-IgG and PGA-IgA in children with acute HSP. These data further indicate that changes in intestinal mucosal immunity were not the major causes for increased PGA-IgA and PGA-IgG, particularly PGA-IgA.

The results of the present study may be limited by several factors. The HSP study population only included patients with non-nephritic HSP, and future studies should include patients with other HSP types in order to investigate HSP pathogenesis. Additionally, the levels of PGA and CICs were not determined in tissue samples due to a lack of clinical samples. Finally, as this was a single-centre study with a relatively small study population, the results may not be generalisable to the wider population. Further studies are required to validate the present results.

In summary, elevated anti-PGA antibodies, particularly PGA-IgA and PGA/PGA-IgA CICs, may be diagnostic factors for acute HSP in children. Food intolerance was not found to be associated with increased levels of serum anti-PGA antibodies in children with HSP.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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