Article

Elevated Levels of Serum IgA against *Saccharomyces cerevisiae* Mannan in Patients with Rheumatoid Arthritis

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This study was undertaken to investigate whether levels of anti-*Saccharomyces cerevisiae* mannan antibodies (ASCMA) in human sera, a marker for several autoimmune diseases, correlate with rheumatoid arthritis (RA). ASCMA-IgA, -IgG and -IgM levels were measured with enzyme linked immunosorbent assays (ELISA) in patients with RA (n = 30) and 152 healthy adult controls. ASCMA-IgA prevalence was significantly higher in RA patients (40%) than in healthy subjects (5.3%). A strong correlation between levels of ASCMA-IgA and CRP ($r = 0.695$; $p < 0.01$) and ESR ($r = 0.708$; $p < 0.01$) in RA patients was observed. No significant differences in ASCMA-IgG or IgM levels were noted between RA patients and healthy control subjects in the present study. This result differs from previous reports. It remains to be evaluated whether elevated ASCMA-levels are common to all rheumatic disorders. *Cellular & Molecular Immunology*. 2009;6(5):361-366.

Key Words: autoantibody, α-1,3-mannan, autoimmune diseases, RA

Introduction

Anti-*Saccharomyces cerevisiae* mannan antibodies (ASCMA) are considered a serological marker for Crohn's disease (CD), a chronic inflammatory disorder of the intestine in which a variety of immune abnormalities have been described at both the systemic and the intestinal levels. ASCMA, together with perinuclear anti-neutrophil cytoplasmic antibodies, can discriminate between CD and ulcerative colitis (1-3). Interestingly, elevated levels of ASCMA have also been found in patients with Behcet's disease, autoimmune hepatitis, systemic lupus erythematosus (SLE) and seronegative spondyloarthritis (4-9). All these disorders seem to be linked to gut pathology, which is underlined by the fact that arthritis is an extraintestinal manifestation of CD (10, 11). Furthermore, rheumatic diseases occur in patients with gluten-enteropathy more frequently than in the general population (12-13). The elevated prevalence of ASCMA in patients with Behcet's disease, SLE and segregative spondyloarthritis also suggests a possibility that it is a phenomenon common to all rheumatic disorders (4, 7-9). It is possible that a higher prevalence of ASCMA might also be seen in patients with rheumatoid arthritis (RA). Notably 2 previous studies did not detect a correlation between ASCMA and RA (7, 8). The present study was undertaken to reevaluate whether ASCMA are elevated in patients with RA.

Materials and Methods

Human subjects and blood samples

Serum samples were collected from 152 healthy volunteers. An equal proportion mixture of sera from all 152 donors was prepared and employed as a negative control sample. Blood samples were collected from a group of 30 hospitalized patients with RA, meeting the American College of Rheumatology 1987 criteria for RA (14). These RA patients attended the Department of Rheumatology and Immunology, Peking University People's Hospital, Beijing between 2006 and 2007. Their disease was in an active phase as defined by the criteria of the Chinese Association for Rheumatology; at the time of the blood collection their condition satisfied 4 of the 5 following criteria: 1) moderate resting pain; 2) morning stiffness ≥1 hour; 3) swelling of 3 or more joints; 4) ≥ 5 tender joints; 5) ESR (Westergren's method) ≥ 28 mm/h. Among the 30 RA patients, 20 (66.7%) were positive for rheumatoid factors (RFs). Average DAS score of these RA patients was 3.7 ± 1.7 (range 2.3-5.5). For steroid treatment, prednisone was administered (2.5-30 mg, median dose 14.3 mg, QD). For DMARD treatment, methotrexate (10 mg, QW), hydroxychloroquine (0.2 mg Bid) and/or leftunomide

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Table 1. Subject groups and their ASCMA prevalence[a]

Subject groups	NS	RA
Number of subjects	152	30
Mean age, year (range)	30.7 (20 - 50)	52.1 (26 - 82)
Female/male	85/67	21/9
Duration of disease, year (range)	-	5.27 (0.2 - 17)
ASCMA-IgA	5.3% (8/152)[b]	40% (12/30) [b] $p < 0.0001$ [c]
ASCMA-IgG	8.5% (13/152)[b]	20% (6/30) [b] $p = 0.062$ [c]
ASCMA-IgM	5.3% (8/152)[b]	13.3% (4/30) [b] $p = 0.122$ [c]

[a]A summary of the data showing the prevalence (percent positive) of ASCMA in normal healthy subjects (NS) and patients with RA. The samples with binding indices above the cutoff values were scored as positive.
[b]The percentage (number of positive/total number in group) of subjects positive for ASCMA Abs are shown.
[c]Two-sides *Fisher's exact* tests were performed comparing ASCMA prevalence of patient groups with healthy adults and the *p* values are shown.

(10 mg Bid) were used.

The blood samples were processed within 18 hours of collection and the sera stored at -80°C until use. Table 1 shows patient and healthy subject characteristics. This study was reviewed and approved by the Ethics Committee of Peking University Health Science Center.

Polysaccharide-based ELISA

Mannan (α-1,3-mannan, mannan of *Saccharomyces cerevisiae*, M7504), D-mannose, dextran (α-1,6-glucan) and Laminarin (β-1,3-glucan) were purchased from Sigma (USA).

Mannan-specific IgG, IgM or IgA antibodies were measured by ELISA. Flat bottom 96-well microtitre plates (Corning- Costar) were coated with 50 μg/ml mannan in 0.1 M PBS (pH 9.6) at 4°C overnight. The plates were washed with 0.05% Tween 20 (Sigma, St. Louis, Mo. USA) in PBS three times between each stage. Each plate was blocked with 10% FCS in PBS for 2 h at 37°C. Serum samples were diluted 1:200 in 2% FCS in PBS and incubated in the ELISA wells for 2 h at 37°C. Detection of IgM, IgG or IgA was done using goat anti-human IgM, IgG or IgA coupled to Horseradish peroxidase (Southern Biotechnology Associates Inc., Birmingham, Al. USA) diluted 1:4000 in PBS-Tween and incubated for 1 h at 37°C. The reaction was developed with 100 μl of O-phenylenediamine (OPD, Sigma) for 5 min and stopped with 100 μl 3M H_2SO_4. Optical density (OD) was measured at 492 nm using an ELISA spectrophotometer (Titertek Multiscan Plus MK II; ICN Flow Laboratories, Irvine, UK). The OD492 nm reading of the control sample on each plate, controlled at 0.2-0.3 throughout the study, was used to calculate the binding index (BI) of the specimen: BI = Sample OD492nm / Control OD492 nm.

Statistical analysis

All serum samples were assayed for ASCMA IgG, IgA and IgM at least 3 times with consistent results. Comparison of the groups was performed using the *Mann-Whitney U* test, while differences in the prevalence of the antibodies under study were analyzed by *Fisher's exact* test in the disease groups and in controls. Correlation between ASCMA levels and RA activity was calculated using *Spearman* correlation analysis. Significance was defined as a p value of < 0.05. A receiver operating characteristics (ROC) curve was generated by plotting sensitivity (y axis) against 1-specificity (x axis). Statistical analysis was performed using SPSS software.

Results

ASCMA in healthy human adults

Serum samples from the 152 healthy volunteers were screened for ASCMA-IgA, IgM and IgG using *S. cerevisiae* mannan-based ELISAs, very few were high responders for IgM and IgA against mannan, but 13 out of the 152 samples (8.5%) had raised ASCMA-IgG levels compared with the rest of the group (Figure 1A, Table 1). Titration of 2 IgG high responder (No. 136; No. 75) and two low responder (No. 115; No. 79) sera confirmed these results (Figure 1B). In subsequent experiments, all serum samples, unless otherwise specified, were 1 : 200 diluted which is in the linear range of the ELISA. In a competition assay, *S. cerevisiae* mannan, but not mannose or glucan polysaccharides (laminarin and dextran), was able to block the binding of antibodies to mannan coated on the ELISA plate (Figure 1C).

Elevated ASCMA-IgA in RA

As shown in Figure 2A, the levels of ASCMA-IgA in serum samples from RA patients were, overall, significantly higher than that in healthy subjects (*Mann-Whitney U* test, $p < 0.0001$). ASCMA prevalence was calculated using the cutoff values defined by the ROC curve (see below). Table 1 shows that ASCMA-IgA prevalence was significantly higher in patients with RA (40%) than in healthy controls (5.3%) ($p < 0.0001$). These results remained significant when ASCMA-IgA levels were corrected for total IgA levels. Further analysis of serially-diluted serum samples from representative RA patients (one strong and one moderate responder) confirmed the screening results (Figure 2B). An increase of ASCMA-IgG levels was also observed in RA patients compared with healthy subjects (20% *vs* 8.5%), although statistical significance was not reached (Table 1).

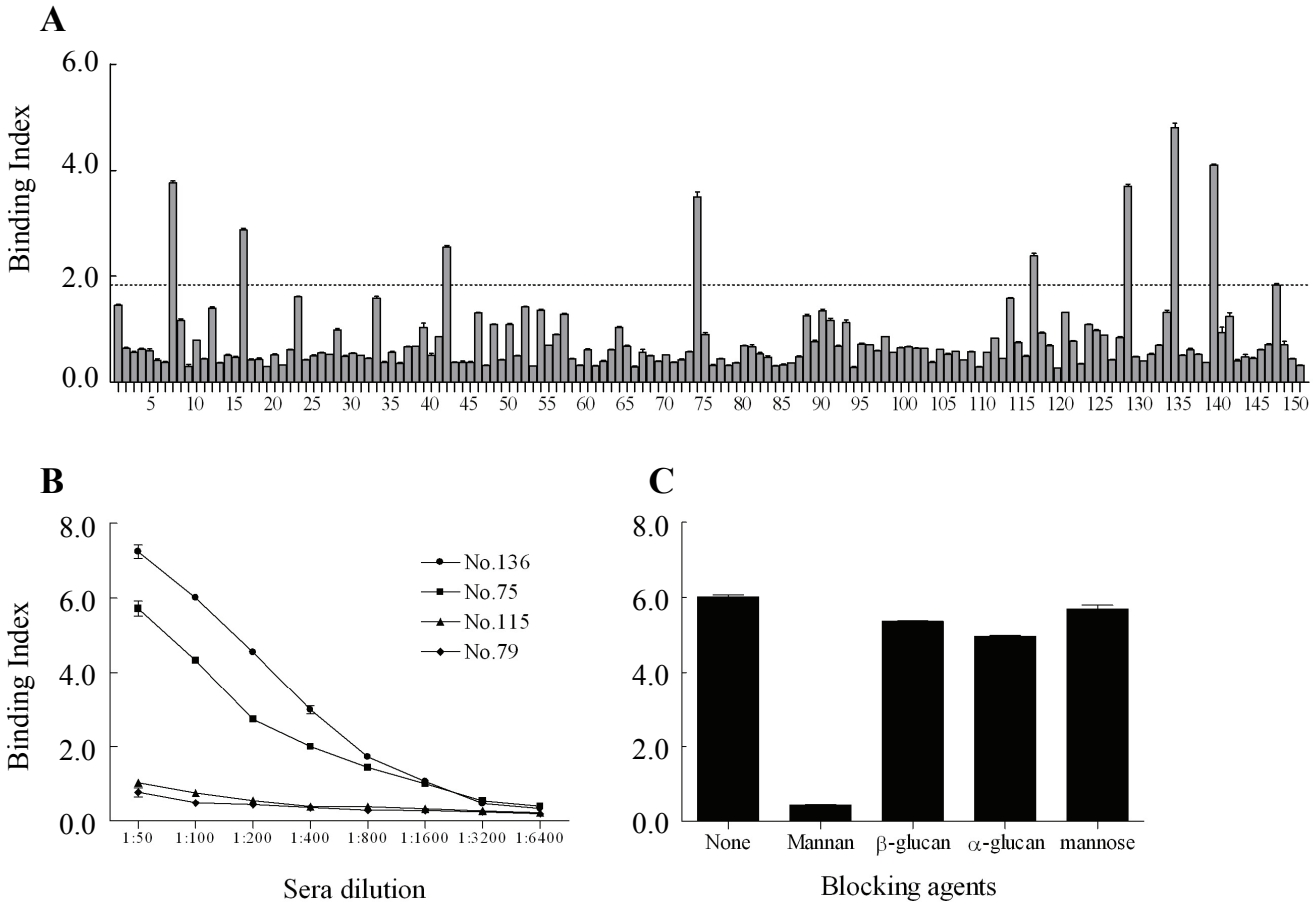

Figure 1. Specificity and sensitivity of the mannan-based ELISA. Serum samples (1:200 dilution, dispensed in triplicates) from 152 healthy adults were individually assayed in ELISAs using polyvinyl plates coated with mannan (A). The detection antibodies were HRP-conjugated goat-anti-human IgG with OPD as substrate. The OD at 492nm was measured and the results calculated as BI using the negative control serum as reference. The horizontal line represents the cutoff value calculated using the ROC curve. Serially-diluted serum samples from representative healthy adults (2 high responders and 2 low responders) were assayed using the mannan-based ELISA (B). In competition assays, human serum samples were pre-incubated with mannan, mannose and α- or β-glucans at 1 mg/ml for 1 h at room temperature and then assayed in ELISAs using plates coated with mannan (C). Samples were dispensed in triplicate wells and the results are expressed as mean ± standard error of the mean (SEM).

ASCMA-IgM levels in patients with RA (13.3%) were not significantly higher than in healthy subjects ($p = 0.122$).

ASCMA-IgA correlation with inflammatory status

In RA, systemic inflammation is a good indicator of the disease activity. As illustrated in Figures 3A and 3B, ASCMA-IgA levels strongly correlated with serum C reactive protein (CRP) levels ($r = 0.695$; $p < 0.01$) and also erythrocyte sedimentation rate (ESR) ($r = 0.708$; $p < 0.01$) in patients with RA (*Spearman* correlation analysis). There was no significant association between RFs and ASCMA in RA sera (Figure 3C). Additionally, removal of RFs, using human IgG-coated particles, from the RF-positive sera of exemplary RA patients did not affect the ELISA results (data not shown).

ROC curve analysis for ASCMA-IgA

ROC curves were plotted for ASCMA-IgA to evaluate sensitivity and specificity of our mannan-based ELISAs in RA. We also performed ROC curve analysis to determine the optimal cutoff for ASCMA in RA. Healthy controls were used as controls (Figure 4). With a cut off value at 1.88 the sensitivity for detecting RA is 40%, while the specificity is 94.7%.

Discussion

For the first time our results show that ASCMA-IgA levels are significantly elevated in patients with RA, and correlate with disease activity as indicated by the ESR/CRP levels (Figures 2 and 3, Table 1), thereby providing a novel and informative serological marker for RA. RFs are one of the formal ACR classification criteria for RA and have prognostic value for the disease (15). However, an

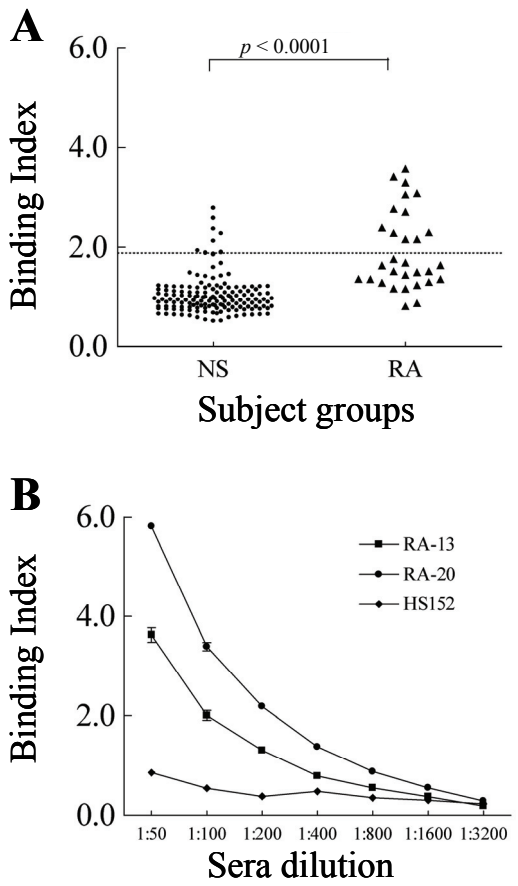

Figure 2. Comparison of ASCMA-IgA levels in different groups. Serum samples from normal healthy adult subjects (NS, n = 152) and patients with RA (n = 30) were individually assayed for ASCMA-IgA (A). The OD at 492 nm was measured and the reading for the negative control serum controlled at 0.2 - 0.3. The results were calculated as binding indices and the mean binding indices of the 3 wells are shown. Horizontal lines represent cutoff values calculated using the ROC curves. *Mann-Whitney U* test was performed comparing RA patient group with the healthy controls and the *p* values are given in the figure. Serum samples from representative RA (B) patients were serially diluted and assayed in parallel experiments.

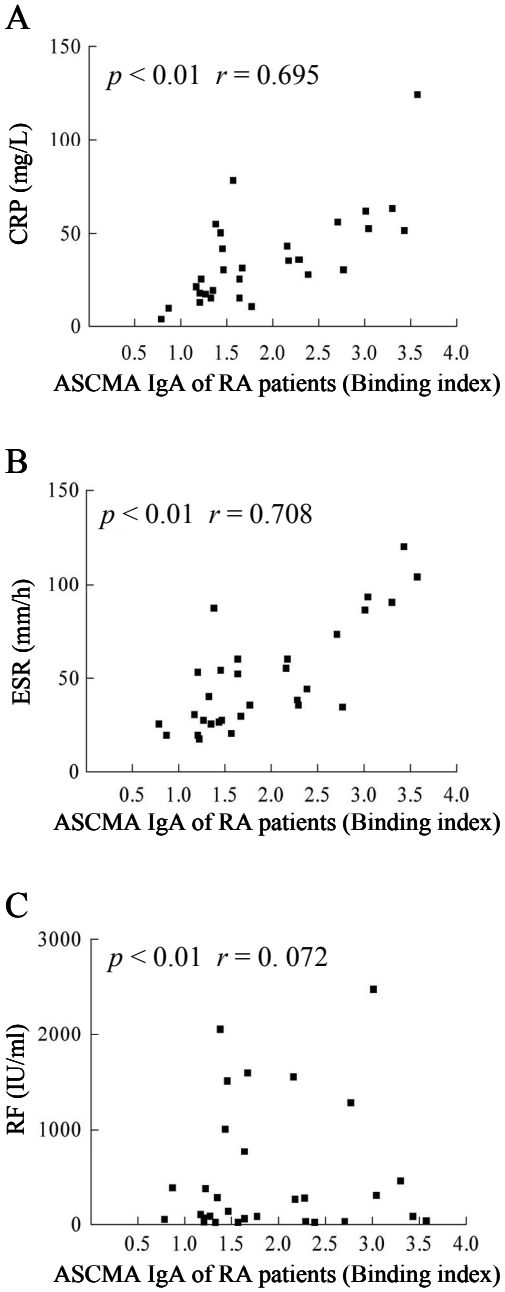

Figure 3. Correlation between ASCMA-IgA levels and disease activity of RA. The ASCMA-IgA levels (mannan-binding index) of sera from RA patients (n = 30) were plotted against their CRP (mg/L), ESR (mm/h) and RF (IU/ml) levels (A, B and C, respectively). The correlation between ASCMA-IgA and CRP, ESR or RF was calculated using *Spearman* correlation analysis and the *r* and *p* values are shown in the figure.

association between RF titers and ESR/CRP of the patients was not observed in this study (Figure 3C). A possible explanation is that all patients enrolled in this study were ward patients in an active phase of disease, while previous investigators compared RF titers in patients of different disease stages.

Two groups have previously examined ASCMA prevalence in RA patients but failed to show a significant correlation (7, 8). There are several possibilities, but not necessarily mutually exclusive explanations for these discrepancies. Firstly, the relatively small sample size in these studies may be partially responsible for the inconsistency. For example, the data reported by Riente et al (8) actually showed a substantial increase in the prevalence of ASCMA-IgA in 79 RA patients compared with 78 healthy controls (17.7% *vs* 8.9%). Although these authors did not come to a positive conclusion for correlation using *Fisher's*

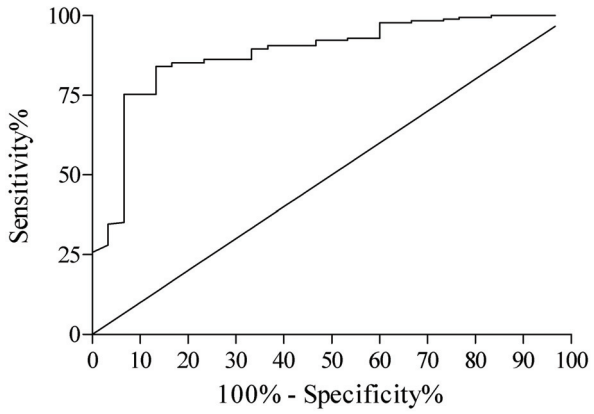

Figure 4. ROC curves for ASCMA-IgA. These curves were obtained by plotting sensitivity for detecting RA against specificity. Healthy subjects were used as the control group.

exact test, our recalculation of their data produced a *p* value of 0.02 when comparing antibody levels of RA patients and healthy subjects by *Student t* test (as employed by authors), which supports a statistically significant difference between the 2 groups if significance is defined as a *p* value of < 0.05. Further studies on larger populations of various ethnic backgrounds are necessary to draw more definitive conclusions. It should also be possible to combine and analyze data from different laboratories provided that standardized ELISA protocols are adopted by all investigators. Additionally, although the patients included in these studies fulfilled the same RA diagnostic criteria, the different genetic background may influence their ability to generate ASCMA, which is supported by the fact that elevated ASCMA have been reported in 20% of healthy relatives of patients with CD compared with 8% in randomly selected healthy controls (11, 12). Finally, the assays used to measure ASCMA are not identical. It is presently not possible to exclude the possibility that these assays perform differently in different disease groups.

Several different mechanisms may explain the elevated production of ASCMA in patients with rheumatic disorders or other autoimmune diseases. Although mannan is a typical pathogen-associated molecular pattern (PAMP) commonly found in both pathogenic and commensal microorganisms (16), it is also part of the glycosylation moieties of *N*-linked glycoproteins in humans and other animals (17). It could lead to production of anti-mannan autoantibodies under certain circumstances. Alternatively, autoantigens without mannan moieties might also be able to elicit antibodies crossreactive with mannan. In our competition/inhibition ELISAs, dsDNA was unable to compete with mannan for binding with ASCMA (not shown), but this does not rule out the possibility that ASCMA are specific for yet unknown autoantigen(s). Thirdly, chances of clinical or subclinical infection in patients with autoimmune disorders by microorganisms might be higher than healthy subjects. Persistence of an infectious agent has been implicated in various forms of arthritis. For instance, reactive arthritis is an acute synovitis in which joint inflammation begins several weeks after acute bacterial diarrhea or urogenital infection (18). A role for bacterial infection in RA is suggested by the reported efficacy of tetracycline antibiotics to suppress synovitis in clinical trials (19).

Previous studies have found a higher prevalence of ASCMA-IgA, but not IgG, in patients with seronegative spondyloarthritis and Behcet's disease (4, 7). Increased production of circulating IgA (as well as IgG) against α-fodrin and C1q in patients with Sjögren's syndrome and rheumatoid vasculitis, respectively, has also been documented (20, 21). These observations imply possible involvement of mucosa-associated lymphoid tissues in production of manna-specific Abs in RA patients. Additionally, imbalance of cytokines or helper T cell subsets accompanying these disorders may also be responsible, as cytokines have profound and pleiotropic effects on B cell proliferation and differentiation. For instance, transforming growth factor β and IL-10 cooperate to favor IgA production.

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