Antiendothelial Cell Antibodies in Patients with Wegener's Granulomatosis: Prevalence and Correlation with Disease Activity and Manifestations

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ABSTRACT. ***Objective.*** Previous studies in small cohorts of patients with Wegener's granulomatosis (WG) or antineutrophil cytoplasmic antibody (ANCA) associated vasculitis have yielded conflicting data regarding the prevalence of antiendothelial cell antibodies (AECA), ranging from 8% to 100%, and the use of AECA as a measure of disease activity. We examined a large, well-characterized cohort of patients with WG and active disease for the presence of AECA.

Methods. Serum from subjects with WG who participated in a clinical therapeutic trial was collected at baseline, when all subjects had active disease. Clinical manifestations and disease activity were documented using the Birmingham Vasculitis Activity Score for WG (BVAS/WG). Serum AECA (IgG) was measured by cyto-ELISA using unfixed human umbilical vein endothelial cells (HUVEC). The AECA positivity cutoff was determined using 71 healthy control samples. Statistical analyses utilized Student's t test, chi-square and Fisher's exact tests, and linear regression.

Results. AECA were detected in 34 of 173 (20%) evaluated serum samples. Mean BVAS/WG did not differ between patients with (7.3 ± 3.2) or without AECA (7.0 ± 3.3) ($p = 0.58$). Among the 34 patients positive for AECA, the antibody titer did not correlate with disease activity (BVAS/WG; $r = 0.09$, $p = 0.60$). There were no statistically significant differences in the frequency of major clinical manifestations between patients with or without AECA.

Conclusion. AECA, as measured using HUVEC, are not highly prevalent among patients with active WG, are not associated with specific clinical manifestations, and do not correlate with level of disease activity. (First Release April 15 2007; J Rheumatol 2007;34:1027–31)

Key Indexing Terms:
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Wegener's granulomatosis (WG) is a small vessel vasculitis with manifestations including upper airway, pulmonary, renal, neurologic systems, and thrombosis. Histological examination of involved tissue has revealed endothelial cell damage[1], and antiendothelial cell antibodies (AECA) have been isolated in the peripheral blood of patients with vasculitis and other autoimmune diseases[2-5]. It has been suggested that AECA could be used as a marker of disease activity in patients with WG[5-11]. There is a strong need to identify biomarkers that can be used to detect active disease, monitor response to therapy, and predict disease flare. We examined the prevalence and clinical association of AECA in a large cohort of patients with WG during a period of active disease.

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MATERIALS AND METHODS

Study subjects and clinical assessment. Study subjects were drawn from participants is the Wegener's Granulomatosis Etanercept Trial (WGET). WGET was a randomized, double-blind, placebo-controlled trial of standard therapy with the addition of etanercept or placebo for patients with active WG. Details of the study design and primary results of the WGET have been reported[12-14]. One hundred eighty study subjects were enrolled in the clinical trial during a period of active disease. Disease activity and specific organ system involvement were assessed using Birmingham Vasculitis Activity Score for WG (BVAS/WG)[13-15]. All subjects had a BVAS/WG ≥ 3 at baseline. BVAS baseline data were available for 170 of the 173 subjects who had AECA investigated. One hundred fifty-six of the 180 (87%) subjects tested positive for antineutrophil cytoplasm antibodies (ANCA)[12,14]. Serum specimens were collected from subjects at their baseline trial visit. The specimens were stored at –80°C and shipped on dry ice.

AECA IgG measurement. AECA were evaluated in 173 of the 180 subjects enrolled in the trial; for the remaining 7 individuals, no serum sample was available. AECA IgG was detected using a cyto-ELISA with unfixed, second passage human umbilical vein endothelial cells (HUVEC) according to a published technique[16]. HUVEC were seeded in a 96-well micro titer plate that was coated in gelatin and allowed to grow to confluence for 24–48 h. They were then washed with Hanks balanced salt solution (HBSS). Nonspecific binding was inhibited by incubating the cells with blocking buffer (HBSS/0.5% BSA) for 60 min at 37°C. After additional washing, HUVEC were exposed to the samples, diluted 1:100, at room temperature for 1 h. Cells were washed again and incubated with alkaline phosphatase-conjugated goat anti-human (Sigma Chemical, St. Louis, MO, USA) for 1 h at room temperature followed by 3 washes. The substrate p-nitrophenylphosphate disodium was added to obtain proper color reaction. After 20 min, the optical density (OD) was read at 405 nm in an ELISA plate reader (BIO-RAD). Each run included a positive and negative control. Samples were run in duplicate and "net" OD values were obtained by subtracting the mean OD readings of blank wells. Positivity or negativity of AECA IgG was determined using 1 standard deviation (SD) of 71 healthy control samples results.

Statistical analysis. Disease manifestations were evaluated and documented using BVAS/WG with specific manifestations being assigned numeric values. The numeric values were combined to develop of BVAS/WG score of 0–68. Analysis comparing AECA positivity and titers was done utilizing Student's t tests, chi-square analysis, and, when appropriate, Fisher's exact tests, and linear regression using SAS Statistical Software for Windows, version 9.1 (SAS Institute Inc., Cary, NC, USA). For all statistical analyses, a 2-tailed $p < 0.05$ was considered significant.

RESULTS

Thirty-four of the 173 (20%) patients who had available baseline serum, all with active disease, were positive for AECA. The 34 AECA-positive patients had a mean BVAS/WG score of 7.3 ± 3.2 and the 139 AECA-negative patients had a mean BVAS/WG score of 7.0 ± 3.3, ($p = 0.58$, Figure 1). In addition, when considering the only 34 subjects that tested positive for AECA, there was no evidence that disease activity measured using BVAS/WG correlated with AECA titers ($r = –0.09$, $p = 0.60$, Figure 2).

There was also no correlation between specific disease manifestations and AECA positivity. The frequencies of organ or system involvements, as defined by the BVAS/WG, for AECA-positive and AECA-negative individuals were as follows: general, 74% versus 71% ($p = 0.73$); cutaneous, 21% versus 19% ($p = 0.88$); mucous membranes/eyes, 35% versus 24% ($p = 0.07$), ear, nose, and throat, 85% versus 75% ($p = 0.19$), cardiovascular, 0% versus 1% ($p = 1.0$); gastrointestinal, 0% versus 1% ($p = 1.0$); pulmonary, 59% versus 60% ($p = 0.086$); renal, 41% versus 55% ($p = 0.16$); and nervous, 9% versus 9% ($p = 1.0$). Among the 34 AECA-positive individuals, only the presence of mouth ulcers demonstrated a statistically significant difference according to AECA status (21% versus 7%, $p = 0.047$).

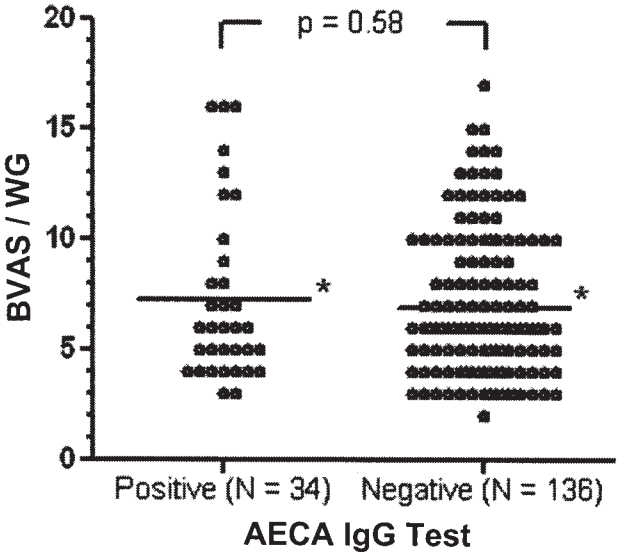

Figure 1. Antiendothelial cell antibodies (AECA) status and Birmingham Vasculitis Activity Score for Wegener's granulomatosis (BVAS/WG) scores among 170 subjects with active WG. *Mean values.

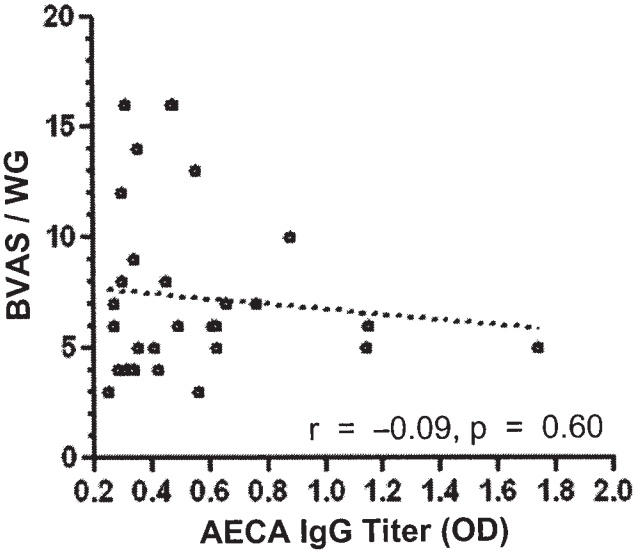

Figure 2. BVAS/WG Scores and AECA titers among the 34 subjects with active WG who tested positive for AECA. OD: optical density.

DISCUSSION

Our study demonstrated that AECA, as measured using HUVEC, neither are highly prevalent among patients with

WG (20%), nor correlate with disease activity or major clinical manifestations. To date, the current study involved the largest cohort of well-characterized patients with WG with prospectively collected data in which AECA were evaluated. All patients had active disease and the disease activity and clinical manifestations were recorded using a standardized technique. The clinical utility of AECA testing has been previously addressed in smaller cohorts, where the prevalence ranged from 8% to 100%[2,7-11,23,24]. These studies are summarized in Table 1.

There are many reasons to believe that AECA might play a role in the pathogenesis of WG and, therefore be useful as a biomarker. Endothelial cells and antibodies to endothelial cells have been implicated in the pathophysiology of vascular injury associated with WG[1]. Endothelial cells and cell fragments have been found in the peripheral circulation of patients with ANCA-associated vasculitis with endothelial cell number correlating with disease activity. Both prevalence and titers of AECA were higher among patients with vasculitis compared to healthy controls or patients with other medical conditions[17,18]. AECA isolated from patients with WG have been reported to alter endothelial cell function *in vitro*[10,19]. When AECA IgG from patients with WG are cultured with HUVEC, there is an upregulation of E-selectin and other adhesion molecules, which causes increases in leukocyte activation and chemokine production[20,21], processes thought to either be causal of or result from vascular injury. Injecting mice with IgG AECA from patients with WG resulted in ANCA production as well as histological vasculitic lesions in the lung and kidney[21].

AECA positivity was seen in 20% of the patients in our study, a prevalence that is different from those in several previous studies[2,8-10,23,24]. Possible explanations for this difference would include those related to study design. Sample size could explain some of the differences, with a greater prevalence of AECA seen in the smaller cohorts (see Table 1). Additionally, insufficient numbers of controls used to set the fold-cutoff for the ELISA detection of AECA could influence results. Further, heterogeneity in the type of vasculitis in the study population could affect the prevalence in studies. Savage, *et al*[2] reported that 59% of the 168 patients with WG or microscopic polyangiitis (MPA) had AECA IgG present. If prevalence of AECA is higher in patients with MPA, this could account for the higher prevalence than seen in this study.

Differences in the specific laboratory methodologies used to measure AECA are another potential source of variation in the measured prevalence of AECA in WG. Although our study and all prior studies appear to utilize similar techniques, small but significant differences were present that could add to the variability of the results. First, previous studies varied in the use of pooled versus single-donor source of HUVEC. The source of HUVEC (single vs pooled) used could affect AECA detection because of endothelial cell membrane antigen variability. Second, prior studies have varied in their use of fixed versus unfixed HUVEC as a substrate for AECA detection. Fixation of HUVEC would result in permeability of cells and the potential for nonspecific binding to internal proteins that could result in false positive results. Third, different dilutions of serum samples and different ELISA cutoffs for AECA detection could also contribute to the different prevalences reported. Finally, variations in the immunofluorescent technique used to detect AECA could alter results including the use of secondary antibodies for immunofluorescence that vary in intensity of fluorescence and different durations of fluorescence (e.g., photobleaching and loss of signal).

The methods used in our study included the use of pooled HUVEC (increasing the sensitivity for AECA detection), unfixed HUVEC (increasing the specificity by excluding AECA reaction with cytoplasmic/nuclear proteins), and a lib-

Table 1. Prevalence of AECA testing and methodologies used in ANCA-associated vasculitis.

	Frampton 1990 [7]*	Ferraro 1990[11]	Savage 1991[2]*	Varagunam 1993[24]	Chan 1993[9]*	Del Papa 1994[10]	Gobel 1996[8]	Holmen 2004[23]	Present Study
Total no. of patients with WG/MPA	14 WG 13 MPA	5 WG	168 WG/MPA	27 WG	6 WG 4 MPA	10 WG	32 WG	24 WG	173 WG
Prevalence of ANCA (%)	100	100	100	NA	100	100	97	100	87
Prevalence of AECA IgG (%)	30	30	59	19	80	100	100	8	20
AECA Testing Methodologies Used									
HUVEC preparation	Unfixed	Unfixed	Fixed	Fixed	Unfixed	Fixed	Unfixed	Unfixed	Unfixed
Dilution	1:400	NA	1:1000	1:20	1:400	1:25	1:100	Variable	1:100
Positivity cutoff	95th percentile of controls mean	2 SD above controls mean	3 SD above controls mean	95th percentile of controls mean	95th percentile of controls mean	NA	3 SD above controls mean	NA	1 SD above controls mean

* These studies did not separate AECA results based on WG or MPA diagnosis. AECA: antiendothelial cell antibodies: ANCA: antineutrophil cytoplasmic antibodies; WG: Wegener's granulomatosis; MPA: microscopic polyangiitis; NA: not available; SD: standard deviation.

eral cutoff of 1 SD for ELISA detection. Despite the increased detection expected by a 1 SD cutoff for the ELISA, only a 20% prevalence was detected in this large cohort of well-characterized patients with active WG.

It is possible that HUVEC is not the optimum substrate for detection of AECA in patients with WG. There is evidence that there are different targets of AECA depending on vessel size[18,22] or organ involvement[23]. Because WG is a small vessel vasculitis that especially targets the pulmonary, renal, and neurological systems and has been recently associated with thrombosis[25] organ-specific endothelial cells or human microvascular endothelial cells may be more suitable targets and should be the focus of further studies.

Our study provides evidence that AECA, detected using the cyto-ELISA HUVEC assay, have a low prevalence (20%) among patients with WG during a period of active disease. Further, the presence of AECA does not correlate with any major specific disease manifestations. AECA detection by the methods employed in our study does not appear to have a clinical role in the management of patients with WG. Further investigations using alternative laboratory procedures to detect organ-specific endothelial antigens seem warranted to best explore the true prevalence and clinical utility of AECA in WG.

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