Complement and Cell Mediated Cytotoxicity by Antiendothelial Cell Antibodies in Takayasu's Arteritis

NARESH KUMAR TRIPATHY, SUNDEEP UPADHYAYA, NAKUL SINHA, and SONIYA NITYANAND

ABSTRACT. ***Objective.*** To study complement and cell mediated cytotoxicity by antiendothelial cell antibodies (AECA) in Takayasu's arteritis (TA).

Methods. Complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC) of AECA positive/negative TA sera were investigated by colorimetric MTT and ^{51}Cr release assays, respectively, using human umbilical vein endothelial cells (HUVEC) as targets.

Results. Seven of 12 (58%) sera positive for IgG and/or IgM AECA exhibited CDC in comparison to none of the 13 AECA negative sera ($p = 0.0052$). The median value of CDC of the AECA positive group was 14% (range 13–21%) and that of the AECA negative group was 1% ($p = 0.0012$). Interleukin 1ß (10 U/ml) treatment of HUVEC resulted in enhancement in CDC of 6 of the 7 AECA positive cytotoxic sera, the median enhancement being 17% (range 7–29%). Tumor necrosis factor-α (100 U/ml) treatment of the targets resulted in a median enhancement by 36% (range 25–55%) in the CDC of 3 of these 7 sera. No sera exhibited ADCC at any of the effector:target ratios tested (10:1 to 100:1).

Conclusion. AECA in TA mediate CDC against endothelial cells and may have a pathogenic role in the perpetuation of vascular damage in this disease. (J Rheumatol 2001;28:805-8)

Key Indexing Terms:
ANTIENDOTHELIAL CELL ANTIBODIES COMPLEMENT DEPENDENT CYTOTOXICITY
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Takayasu's arteritis (TA) is a large vessel vasculitis characterized by the inflammation of large elastic arteries, mainly the aorta, its major branches, and pulmonary and coronary arteries. It has a strong predilection for women under 40 years of age[1,2]. TA has become increasingly recognized as a worldwide entity but the majority of cases have been reported from Japan, Southeast Asia, and Africa[1,3]. It is the most common primary vasculitis and a major cause of renovascular hypertension in the young population in India[4]. The etiopathogenesis of the disease is not well understood. However, most current clinical and laboratory data point to the role of autoimmune mechanisms comprising both humoral and cellular pathways in its pathogenesis[3,5].

We and another group have observed the presence of antiendothelial cell antibodies (AECA) in a significant proportion of patients with TA[6,7]. We also observed that patients had higher levels of IgG and IgM AECA compared to controls, and there was a significant association between IgG AECA and disease activity. AECA have been found to cause apoptosis in scleroderma[8], complement dependent cytotoxicity (CDC) in hemolytic-uremic syndrome[9] and Kawasaki disease[10-13], and antibody dependent cellular cytotoxicity (ADCC) in systemic lupus erythematosus (SLE)[14], scleroderma[15,16], and Wegener's granulomatosis[17,18]. However, it is not known whether AECA in patients with TA are pathogenic or simply represent an epiphenomenon of vascular damage.

In patients with TA we investigated whether binding of AECA to constitutive antigenic determinants of endothelial cells causes cytotoxicity to endothelial cells in the presence of complement or peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Subjects. Seventy-one TA sera were screened for IgG and IgM AECA by a cellular ELISA using human umbilical vein endothelial cells (HUVEC). The cutoff limit for a positive value was taken as mean + 2 SD of 100 age and sex matched healthy controls run in parallel. Fifteen samples with the highest titers of IgG and/or IgM AECA and 13 AECA negative samples were selected for study.

All patients fulfilled American Rheumatism Association (ARA) criteria for TA[19] and the disease was classified as type 1, 2, 3, or 4 according to the anatomical involvement[20]. The disease was considered to be in the active stage if 2 or more of the following were present along with other features of the disease: (1) constitutional features like fever, arthralgias, myalgias, weight loss of unknown cause; (2) elevated erythrocyte sedimentation rate (> 30 mm/h); (3) elevated C-reactive protein (> 0.6 mg/dl)[21].

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Culture of human endothelial cells. Endothelial cells were harvested from human umbilical cord vein by collagenase digestion and cultured as described[22]. Only primary cultures of endothelial cells were used as targets in the cytotoxicity assays.

Preparation of effector cells. PBMC were isolated from heparinized venous blood from a single healthy individual by density gradient centrifugation using Histopaque 1.077 (Sigma, St. Louis, MO, USA) following manufacturer's instructions. The harvested cells were washed twice with plain RPMI-1640 medium (Sigma). The purified cells were suspended in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) at a concentration of 1×10^6 cells/ml and incubated in a 25 cm^2 tissue culture flask at 37°C in CO_2 incubator for 2 h to deplete adherent cells. Nonadherent cells were harvested from the flask and resuspended in RPMI-1640 with 10% heat inactivated FBS (C-RPMI) at a concentration of 10×10^6 cells/ml. These cells were used as effectors in the cytotoxicity assay.

Complement dependent cytotoxicity. CDC of AECA was determined by MTT [3-(4,5-dimethylthiazol–2-y1)-2,5-diphenyl tetrazolium bromide] assay as described with some modifications[11,23]. Primary culture endothelial cells were seeded in 96 well flat bottom tissue culture plates (Nunc, Roskilde, Denmark) at a concentration of 4×10^4 cells per well and cultured overnight. Test sera were diluted 1:10 in plain modified Eagle's medium (MEM; Gibco BRL), heat inactivated (56°C for 30 min), and sterilized by filtration through 0.22 μm membrane filters; 100 μl of each sample was added in triplicate wells. The positive control was 1:10 diluted murine anti-human monoclonal antibody W6/32 (Dako, Glostrup, Denmark), which is IgG2a isotype directed against HLA class 1 monomorphic determinants and causes 34 to 57% endothelial cell lysis in the presence of the complement[17]. This Mab also served to check reproducibility of the assay. The plate was incubated at 37°C for 3 h in CO_2 incubator. After 2 washes with plain MEM, 100 μl freshly thawed human AB serum diluted 1:4 with plain MEM was added to each well as a source of complement. 100 μl/well of 3% Triton X-100 and heat inactivated human AB serum were added in 2 sets of triplicate wells to measure MTT uptake by lysed cells (background uptake) or maximum uptake of MTT, respectively. The plate was again incubated as above for 2 h. After 2 washes, 100 μl of 1:4 diluted MTT solution (Sigma) (5 mg/ml) was added in all the wells and the plate was incubated at 37°C for 4 h as above. MTT solution was removed and 100 μl of dimethyl sulfoxide (Sigma) was added in all the wells. The plate was incubated at 37°C for 30 min with intermittent shaking to solubilize the formazan crystals. The plate was read at 570 nm in an ELISA reader (Tecan Spectra, Austria). The percentage cytotoxicity for each sample was calculated using the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Maximum uptake} - \text{sample uptake}}{\text{Maximum uptake} - \text{background uptake}} \times 100$$

The cutoff value of the cytotoxicity of test sera was taken as mean + 2 SD of the cytotoxicity of AECA negative TA sera.

Antibody dependent cellular cytotoxicity. ADCC was determined by ^{51}Cr release assay as described with modifications[17]. The primary cultures of endothelial cells were seeded in 96 well flat bottom tissue culture plates at a concentration of 2×10^4 cells/well and cultured overnight. Endothelial cells were labeled with 5 μCi of ^{51}Cr (sodium chromate, 1 mCi/ml; Barc, Bombay, India) for 4 h at 37°C in CO_2 incubator. The excess of ^{51}Cr was removed and the plate was washed 3 times with plain MEM. Test sera were diluted 1:2, 1:5, 1:10, 1:25, and 1:50 with MEM containing 10% FBS. Diluted sera were then heat inactivated and sterilized by membrane filtration. 100 μl/well MEM with 10% FBS or diluted AECA positive or negative TA sera were added in the plate in triplicates. The plate was incubated 2 h at 37°C in CO_2 incubator. After 2 washes with plain MEM, effectors were added in each test well in a final volume of 200 μl/well of C-RPMI at an effector:target (E:T) ratio of 10:1, 25:1, 50:1, and 100:1. One set of triplicate wells contained 200 μl of 3% Triton X-100 and another set of triplicate wells contained 200 μl/well of RPMI-1640 with 10% FBS to determine maximum and spontaneous release of radioactivity, respectively. Two AECA positive scleroderma sera producing 25–30% and 40–45% of cytotoxicity, respectively, at E:T ratio of 25:1 were used as positive controls in each run to check the reproducibility and validity of the assay. After centrifugation (250 g, 5 min) the plate was incubated 6 h as above. The plate was centrifuged again at 250 g for 5 min, 100 μl of cell-free supernatant was collected from each well, and released radioactivity was measured using a multigamma counter (LKB, Pharmacia, Sweden). The percentage cytotoxicity for each sample was calculated using the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Sample release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

The cutoff value of the cytotoxicity of test sera was taken as mean + 2 SD of the cytotoxicity of AECA negative TA sera.

Cytotoxicity after IL-1ß and TNF-α pretreatment of endothelial cells. AECA positive sera found to be cytotoxic to resting HUVEC were further evaluated for cytotoxicity after cytokine pretreatment of the targets. For this purpose the overnight grown HUVEC were treated with 10 U/ml of IL-1ß or 100 U/ml of TNF-α (both from R&D Systems, Minneapolis, MN, USA) for 6 h at 37°C and the cytotoxicity experiments were carried out as described above.

Statistical analysis. Fisher's exact test was used for testing significance of qualitative data and Mann-Whitney U test for quantitative nonparametric data.

RESULTS

Complement dependent cytotoxicity. Seven of 12 (58%) AECA positive TA sera and none of the 13 AECA negative sera exhibited cytotoxicity ($p = 0.0052$). Of these 7 cytotoxic sera from patients with TA, 4 were positive for both IgG and IgM, 2 were positive for IgM, and one was positive for the IgG isotype of AECA. The median cytotoxic activity of these AECA positive sera (14%, range 13–21%) was significantly higher than that of AECA negative sera (1%; $p = 0.0012$) (Figure 1). These sera were tested in 3 additional experiments and revealed reproducible cytotoxicity

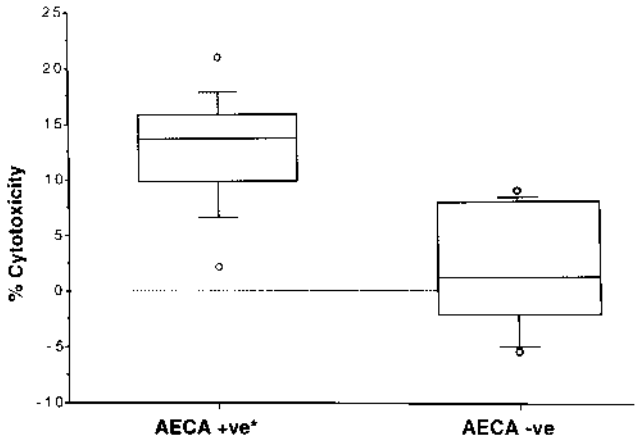

Figure 1. Complement mediated cytotoxicity of AECA positive and negative groups. Horizontal line represents 50th percentile and the vertical line extends from the 10th to 90th percentile. *$p < 0.01$.

with an interassay coefficient of variation of less than 5%. The cytotoxicity produced by murine Mab W6/32 varied from 45% to 59% in all experiments. There was no correlation of the cytotoxicity of AECA with disease activity or with the type of disease.

Antibody dependent cellular cytotoxicity. None of the 15 AECA positive and 10 AECA negative sera showed cytotoxicity at any of the different E:T ratios or at the dilutions of the test sera specified in the assay method. Similar results were obtained in 3 additional experiments with coefficient of variation < 7.5%. The median percentage cytotoxicty of AECA positive and negative sera were comparable, –2.14% and –2.36%, respectively (p = 0.05). The 2 positive sera repeatedly produced a cytotoxicity of 25%–30% and 40%–45%, respectively.

Cytotoxicity after IL-1ß and TNF-α pretreatment of endothelial cells. Six of the 7 AECA positive sera that exhibited CDC showed enhancement in cytotoxicity after IL-1ß pretreatment of HUVEC, and median enhancement was 17% (range 7–29%). TNF-α pretreatment of the targets resulted in a median enhancement by 36% (range 25-55%) in the CDC of 3 of these 7 sera.

DISCUSSION

AECA have been observed by us[6] and others[7] in 37% and 94% of patients with TA, respectively. We also observed a significant association of these autoantibodies with disease activity[6]. This led us to investigate whether AECA have a pathogenic role in the vascular damage in TA, even if they are secondarily formed in response to antigens of endothelial cells released after damage by some other mechanism(s). We investigated 12 IgG and/or IgM AECA positive and 13 AECA negative sera of TA patients for CDC. Seven of 12 (58%) AECA positive sera produced cytotoxicity ranging from 13% to 21% (median 14%), which was enhanced further on cytokine pretreatment of the targets. However, no AECA negative sera was found to be cytotoxic. Although the prevalence of AECA was higher in patients with active disease[6], the AECA of both the active and inactive disease groups exhibited CDC and both had comparable mean cytotoxic activity. An explanation for this may be that the AECA positive patients with inactive TA we observed may actually have histologically active disease. The clinical and laboratory variables used for assessing disease activity in TA do not always reflect histologic activity of the disease. Surgical biopsy specimens from clinically inactive patients revealed histologically active disease in 42–44% of the patients[24,25]. Further, a recent autopsy study in Indian patients with TA has shown the presence of active inflammatory arterial lesions in the seemingly chronic fibrotic phase of the disease[26]. AECA against constitutive antigenic determinants of cultured HUVEC have been found to cause CDC in 93% of patients with hemolytic-uremic syndrome (HUS)[9] and 32 to 52% of patients with Kawasaki disease[10,11]. Leung, *et al*[9] observed that both IgG and IgM AECA present in the sera of patients with HUS cause CDC of resting HUVEC, as we observed. In Kawasaki disease only IgM but not IgG AECA have been found to produce CDC against resting or interferon-γ treated HUVEC, and cytokine pretreatment of the targets significantly enhanced the cytotoxicity of these antibodies[10-12]. However, in another study on Kawasaki disease both IgG and IgM AECA were found to produce CDC against IL-1 and TNF-α stimulated HUVEC, but IgM AECA were more effective[13]. The mean cytotoxic activity of AECA was 25 ± 13% in HUS[9] and 36 ± 14% in Kawasaki disease[10]. In our study the median value of cytotoxicity was 14% against resting HUVEC, with median enhancement of 17% and 36% by IL-1ß and TNF-α pretreatment of HUVEC, respectively. These variations may be due to different cytotoxic potential of these autoantibodies in different diseases or to differences in the source of complement and methodology used. The CDC exhibited by AECA in TA suggest a pathogenic role of these autoantibodies in the disease. Though this perhaps may not be the primary pathogenic mechanism of TA, it may be important in the perpetuation of the vascular damage. There are few data on the pathogenic potential of AECA in TA. Recently a panel of monoclonal AECA derived from a single patient with TA were found to activate HUVEC, as shown by increased secretion of IL-6 and von Willebrand factor and increased expression of adhesion molecules associated with nuclear factor κB activation and increased adhesion of monocytes to these cells[27]. We used AECA negative sera of the same disease group as controls to compare the cytotoxicity of AECA positive sera. In addition, we also investigated AECA positive sera of 7 healthy controls, but none of these exhibited cytotoxicity (unpublished results). This shows that only AECA containing the immunoglobulin fraction of patient sera mediate CDC, and no other serum component. Most of the above studies used radioactive methods for determining CDC. We carried out our cytotoxicity assay by a colorimetric method using MTT, a tetrazolium salt forming dark purple crystals of formazan after reduction by succinate dehydrogenases in living cells[11,23]. This technique is simple and nonradioactive and requires no manipulation of targets before antibody and complement treatments. Furthermore, it evaluates the number of living cells remaining after cytolysis has occurred and provides highly reproducible data. Our interassay coefficient of variation was < 5%.

IgG AECA have been found to induce ADCC of resting HUVEC in SLE[14], scleroderma[15,16], and Wegener's granulomatosis[17,18]. Most of the sera of our AECA positive patients contained both IgG and IgM isotypes. However, we did not observe ADCC at any of the E:T ratios ranging from 10:1 to 100:1 and different dilutions of test sera (1:2 to 1:50). In similar observations, AECA in Kawasaki disease produced CDC but not ADCC[11].

We conclude that patients with Takayasu's arteritis develop antiendothelial cell antibodies against constitutive antigenic determinants on endothelial cells, and their ability to mediate complement dependent cytotoxicity against HUVEC *in vitro* suggests a pathogenic role of these autoantibodies at least in the perpetuation of the vascular damage in TA.

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