Anti-endothelial cell antibodies in Behçet's disease

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ABSTRACT

Objective. *Circulating antibodies that bind to human endothelial cells cultured in vitro have been detected in a variety of diseases, including Behçet's disease. In this disorder the reported prevalence of AECA has varied widely. One likely source of variability is the ELISA assay itself, in which differing conditions and reagents have been used in different reports.*

Methods. *We have re-examined the frequency of AECA in 132 Turkish Behçet's patients and 50 healthy Turkish controls, comparing several different methods of preparing the target endothelial cells. Human umbilical vein endothelial cells (HUVEC) were used either: 1) fresh and non-treated, 2) fixed, or 3) TNFα-stimulated. All stages of the procedures were performed at room temperature.*

Results. *In Behçet's patients, using fresh, non-treated HUVEC, 17 of 130 (13.1%) and 9 of 132 (6.8%) sera were positive for IgG- and IgM-AECA, respectively. However, among 50 normal controls, 2 (4.0%) had IgG-positive and 4 (8.0%) had IgM-positive ELISAs under the same conditions. The difference in the frequency of positives between patients and controls was not statistically significant. Fixed HUVEC and TNFα-treated HUVEC gave similar results as well. When group means were examined, only the mean for IgG-AECA determined with TNFα-stimulated HUVEC reached statistical significance.*

Conclusion. *The discrepancy between our data and earlier reports in the literature probably reflects the methodological differences alluded to, and highlights the difficulties in interpreting ELISA assays for AECA.*

Introduction

Behçet's disease (BD) is a condition of unknown etiology, which is characterized by recurrent orogenital ulceration, skin lesions and an inflammatory arthritis, as well as by vasculitic involvement of the major peripheral arteries and the veins and vessels of the central nervous system (1). The vascular involvement of BD includes thrombophlebitis, seen in about 25% of patients,seldom if ever with embolization, and arteritis with occlusion and aneurysm formation (2). This has suggested the existence of a hypercoagulable state in BD patients, and has raised the possibility of a role for the endothelium in the pathogenesis of BD. Indeed, previous studies in Behçet's disease have been interpreted to show evidence of endothelial dysfunction (3-5), and several studies have found AECA in 18-37% of BD patients (6-12). However, a number of methodological differences exist between these studies, making it difficult to compare their results. Some studies, for example, were performed at 37ºC, a temperature at which surface-bound IgG can be internalized (13), while others have not taken into account the possible occurrence of heterophil antibodies that might have augmented the apparent specific binding in ELISA (14). Such variations have suggested a need for standardized tests for AECAs (15). In the present study, we measured AECA by ELISA in a large group of Behçet's patients and controls, and examined the significance of several variables in the methodology.

Materials and methods

Source of sera

Sera were obtained from 132 Turkish patients with BD attending three centers in Turkey (Cerrahpasa and Istanbul Medical Faculties, University of Istanbul and Gulhane School of Medicine, Ankara) and from 50 healthy Turkish controls. All patients fulfilled the ISG criteria for diagnosis of BD (16). The clinical features of the patients are shown in Table I.

The control group consisted of 22 females and 28 males,with a mean age of 27.7 ± 8.4 years (range 19-49 years).

Table I. Clinical features of 132 patients with BD.

Age (mean, range)	24.8 ± 5.4, 19-42 years
Sex	31 female, 101 male
Oral ulceration	132 (100%)
Genital ulceration	113 (85.6%)
Skin lesions	82 (60.6%)
Arthritis or arthralgia	48 (36.4%)
Eye disease	45 (33.8%)
Thrombosis	32 (24.0%)
Neurological lesions	22 (16.5%)

Endothelial cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (17) and grown in M199 medium (Gibco) supplemented with glutamine, heparin, antibiotics, endothelial cell growth factor and 10% heat-inactivated fetal calf serum (FCS, Gibco). The cells were used at passage 2-4 and seeded onto gelatin-coated 96-well microtiter plates. The cells were used when confluent (usually within 48-72 hr) and were studied under three different conditions: 1) fresh, non-treated, 2) fresh TNFα-stimulated, or 3) non-treated and glutaraldehyde-fixed.

Anti-endothelial cell antibody ELISA

After blocking non-specific binding sites for 1 hr with diluent (M199, containing the supplements enumerated, as well as 3% bovine serum albumin), plates were washed twice with washing buffer (phosphate buffered saline (PBS), containing 1% BSA). An aliquot of 100 µL serum was added to each well at a dilution of 1/25 in the diluent, followed by 1 hr incubation. Each microtiter plate included wells with positive reference sera, wells with diluent alone (including all reagents except study serum) serving as blanks, and 4 normal sera serving as internal controls. All sera were run in triplicate. After the wells were washed 4 times with PBS-BSA they were incubated for 1 hr with alkaline phosphatase-conjugated $F(ab')_2$ of either goat anti-human IgM ($Fc_{5\mu}$ specific) or goat anti-human IgG (Fcγ specific) diluted 1:2000 (Jackson Immunoresearch Lab, Inc., West Grove, PA). After 2 washes with PBS-BSA and 2 more washes with PBS alone, 100 µL of p-nitrophenyl phosphate (Pierce) was added to each well, and absorbance at 410 nm was read in a Dynatech ELISA spectrophotometer when the positive reference serum reached a predetermined level.

For TNFα treatment, plates were incubated at 37ºC for 24 hours with human recombinant TNFα (Sigma), 100 IU per mL medium; this concentration of TNFα was maintained throughout the ELISA determination. For fixation, untreated HUVEC were exposed to 0.1% glutaraldehyde (100 µL/well) for 5 min at 4ºC. All stages of the ELISA were performed at room temperature (22-26ºC) to prevent possible internalization of surface-bound immunoglobulins. Although some morphological changes were evident, HUVEC remained attached and confluent during all stages of the assays.

Results were expressed as a binding index (B.I.), calculated from 410 nm absorbance values as follows:

$$\text{B.I.} = \frac{A_{sample} - A_{blank}}{A_{pos.\ reference} - A_{blank}}$$

Results were considered positive when B.I. values were >2 SD above the mean values obtained for control sera. The cumulative intra- and inter-assay coefficients of variation were < 20%.

Statistical analysis was performed using the software package SPSS 4.5 (SPSS Inc., USA). Data are reported both as the frequency of positive sera and as the mean ± SD of the binding indices. Mann-Whitney U, chi-square, Fisher's exact and Kolmogorov-Smirnov tests were used where appropriate and the alpha value was set to 0.05.

Results

In six experiments among healthy controls (three conditions, two types of antibody - IgG and IgM), examination of the binding index using the Kolmogorov-Smirnov test indicated that data were normally distributed, except in the case of IgG- and IgM-AECA measured on fresh, non-treated EC. Thresholds for positivity ranged between 72.5 and 125.6 BI units in the six experiments.

As shown in Table II, when fresh, non-treated HUVEC were the target, 17 of 130 Behçet sera (13.1%) and 2 of 50 normal sera (4%) were positive for IgG-AECA; this difference approaches, but does not reach, statistical significance. Nine of 132 Behçet sera (6.8%) and 4 of 50 normal sera (8%) were positive for IgM-AECA. Similarly, no statistically significant differences were found between patients and controls using fixed HUVEC or TNFα-stimulated HUVEC. When group means were examined, only the mean for IgG-AECA determined with TNF-α-stimulated HUVEC reached statistical significance (Figs. 1 & 2; Table II).

Table II. Comparison of means and frequency of positive AECAs among BD patients and healthy controls.

		Patients		Controls		p	
		BI (mean ± SD)	Positive (%)	BI (mean ± SD)	Positive (%)	BI[1]	Positives[2]
IgG	Fresh, non-treated	46.0 ± 28.0	17/130 (13.1)	40.4 ± 16.0	2/50 (4.0)	0.373	0.076
	Fixed	62.2 ± 27.0	10/88 (11.4)	60.3 ± 16.0	1/29 (3.4)	0.781	0.288
	Fresh, TNFα-stimulated	61.0 ± 28.1	17/80 (21.3)	44.0 ± 14.5	1/23 (4.3)	0.001	0.068
IgM	Fresh, non-treated	45.8 ± 22.7	9/132 (6.8)	45.1 ± 21.9	4/50 (8.0)	0.673	0.754
	Fixed	60.7 ± 26.0	2/89 (2.2)	66.4 ± 29.6	2/30 (6.7)	0.423	0.263
	Fresh, TNFα-stimulated	47.1 ± 34.5	4/91 (4.4)	50.7 ± 27.0	1/31 (3.2)	0.269	1.000

BI: Binding index, [1]Mann-Whitney U, [2]Chi-square

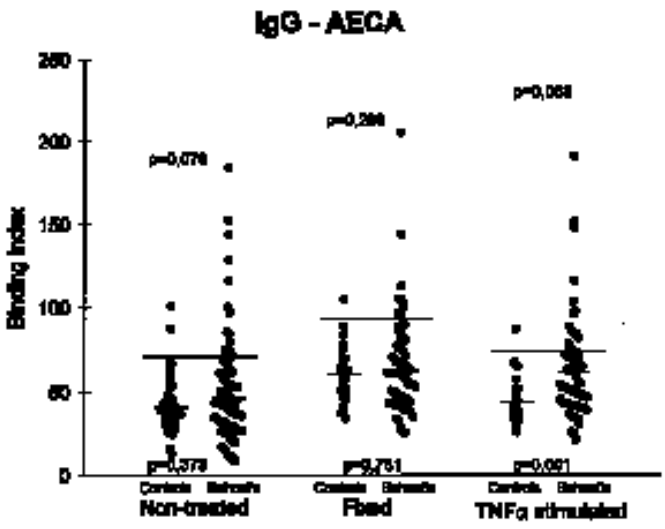

Fig. 1. Binding indices for IgG-AECA. Each dot is the mean for an individual sample assayed in triplicate. The short horizontal lines represent mean values for each group. The longer lines denote the mean +2 SD of healthy controls calculated for each working condition. Upper and lower p values represent statistical comparisons of patients and controls with respect to frequency of positives (Chi-square) and means (Mann-Whitney U), respectively.

The frequency of positives in the Behçet group varied under the different conditions, most noticeably for IgG-AECA, where TNFα-stimulated HUVEC gave results nearly 100% higher than the other two conditions; this difference was not present in the control group (Table II). Comparison of the only identical samples studied under all of the three different working conditions made this observation clearer (Table III): fixation of the HUVEC increased IgG and IgM antibody binding in both patients and controls. However, TNFα-stimulation of HUVEC resulted in an increment of IgG binding in the patient group, but not in the healthy controls.

Of 32 patients with thrombosis, 3 (9.4%) had a positive IgG-AECA and 2 (6.2%) had a positive IgM-AECA. Among 22 Behçet's patients with neuro-parenchymal involvement, two (9.0%) had a positive IgM-AECA and one (4.5%) had a positive IgG-AECA.

Discussion

Antibodies to endothelial cells have been described in a variety of inflammatory and vasculitic disorders and, in some of these, their presence has been related to the pathogenesis (15, 18). Behçet's disease is a systemic vasculitic disorder in which thrombotic complications are more prevalent than in other vasculitides. In search of an etiology, various methods and working conditions have been used to determine AECA in patients with BD (6-12).

Tsukada *et al*. reported that, in 30 patients with neuro-Behçet's disease, antibodies against non-treated rat brain microvascular endothelial cells, determined by ELISA at 37ºC, were increased only in active cases (6). Among studies done with unfixed HUVEC, Pivetti-Pezzi *et al*., using a radioimmunassay method and incubating at RT, found a 13.6% positivity for either IgM- or IgG-AECA in 22 patients (7). Others studies, performed by ELISA in which untreated HUVEC were kept at 37ºC throughout the incubation steps, revealed comparable results. Thus, Aydintug *et al*. detected 11.1% and 9.7% abnormal titers of IgM- and IgG-AECA, respectively, in 72 cases (8), while Cervera *et al.* reported 26% overall positivity in 30 patients (10) and Direskeneli *et al*. found elevated AECAs in 29% of 70 patients (9).

In contrast, Triolo *et al.* who used fixed HUVEC in an ELISA, found 43% positive results in 21 patients (11). As a further complication, studies using fresh microvascular endothelial cells (MEC) kept at 37ºC have yielded higher positive results than studies using HUVEC. Thus, Cervera *et al*. found a 43% overall positivity among 30 patients using human adipose tissue MECs (10), and Lee *et al.* reported 37.4% and 15.3 % positive results, respectively, for IgM- and IgG-AECA against dermal MEC in a group of 131 BD patients (12). In all these studies, attempts to detect functional effects of AECA on endothelial cells have resulted in equivocal or negative results. It is difficult to interpret the variations in frequency of positivity of AECA, at least partly because of the variety of methods used for their detection. The technical pitfalls of the ELISA procedure for AECA deserve comment.

Recently, it was shown that cultured HUVEC can internalize surface-bound IgG by a mechanism inhibited at temperatures below 27ºC (13). Thus, studies done at 37º may reflect this phenomenon. Furthermore, fixation of EC prevents cell detachment throughout the ELISA (15) but, since fixation may lead to some degree of permeabilization of the EC membrane, antibodies binding to intracellular constituents may be included in the reactivity ob-

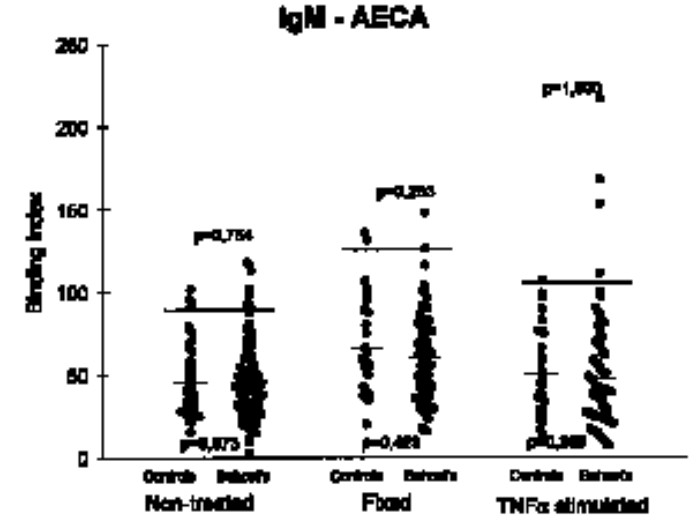

Fig. 2. Binding indices for IgM-AECA. Results depicted in the same manner as in Figure 1.

Table III. Comparison of the means of identical samples under various working conditions.

Compared conditions[1]	Patients (n) IgG-AECA (69)	Patients (n) IgM-AECA (79)	Controls (n) IgG-AECA (19)	Controls (n) IgM-AECA (30)
Non-treated vs. fixed	p = 0.001	p = 0.005	p = 0.001	p < 0.001
Non-treated vs. TNFα-stimulated	p = 0.001	p = 0.093	p = 0.183	p = 0.217

[1]Mann-Whitney U test, Group means were not included.

tained under these conditions (18). It has been reported that antibodies reacting with animal proteins (heterophil antibodies) can augment the results in cellular ELISA experiments. In that report, adding FCS to the diluent was recommended to prevent such interference (14). Endothelial activation by cytokines such as TNFα leads to an array of alterations in EC phenotype, including the upregulation of leukocyte adhesion molecules (19). Since TNFα release probably occurs in BD, as in many other diseases, treatment of EC *in vitro* with TNFα might simulate conditions encountered *in vivo*.

In the present study, we compared three different ELISA conditions for the determination of AECA. We used fresh unfixed EC, kept at room temperature and stabilized by a relatively high concentration of albumin (3% BSA) included in our diluent (20). We also studied the same patient and healthy control sera using fresh unfixed TNFα-stimulated EC and fixed EC. We did not detect any statistical difference in the frequency of AECA between Behçet's patients and healthy controls under any of the three working conditions, although mean IgG-AECA values using TNFα-stimulated HUVEC approached significance. Although a few Behçet patients were positive in one system or another, we found positives among the controls, as well. This finding seems to agree with a recent report on the presence of "natural" AECA in healthy subjects (21). The increase in binding indices seen with TNFα-stimulated EC may be due to changes in the EC phenotype induced by this cytokine or, possibly, to increased surface antigens due to membrane vesiculation (22). On the other hand, circulating levels of soluble adhesion molecules are increased in BD (23), which could result in a certain degree of neutralization of circulating antibodies to surface adhesion molecules, and therefore to their underestimation in the ELISA.

The discrepancy between our results and earlier reports in the literature probably reflects the methodological differences alluded to, and highlights the difficulties in interpreting ELISA assays for AECA. Whether methodological differences may give rise to the disparate results reported for AECAs in other vasculitides requires further study. Nevertheless, our results at least suggest that patients with BD may have circulating antibodies to induced antigens on EC, and thus argue for the possibility that in BD the vascular endothelium is activated.

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