unclear whether AECA play a direct role in causing endothelial dysfunction in BD or merely represent clinical markers of disease activity or progression.

Adherence of leukocytes to vascular EC is regulated by the treatment of biological response modifiers (BRM) to EC.[11,12] The binding of leukocytes to EC is governed by the expression of cell adhesion molecules which are regulated by BRM.[13] T lymphocyte infiltration across the vessel wall is prominent in BD lesions.[14]

In this study, we used enzyme-linked immunosorbent assay (ELISA) and immunofluorescence flow cytometric analysis, which allowed detection of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin on human dermal microvascular endothelial cells (HDMEC) following their incubation with AECA-positive sera of BD patients. In addition, we used an in vitro model of the vascular wall consisting of HDMEC monolayers to study the binding of T lymphocytes to HDMEC after stimulating with AECA-positive sera of BD patients.

MATERIALS AND METHODS

Subjects

Sera were obtained from healthy normal volunteers (n=60), from patients with BD (n=75), each of whom fulfilled the diagnostic criteria of the International study group for BD.[15] All serum specimens were obtained at a time when patients had active disease. Sera were collected and stored at $-70^{\circ}C$ until use.

Cell culture

HDMEC were isolated from human neonatal foreskins by trypsinization and percoll gradient centrifugation as described previously.[16,17] Cells were cultured in endothelial basal media (Clonetics Corp., San Diego, CA, U.S.A.) with epidermal growth factor 5 ng/ml (Clonetics), hydrocortisone acetate 1 mg/ml (Sigma Chemical Co., St. Louis, MD, U.S.A.), dibutyryl cyclic AMP 5×10^{-5}M (Sigma), penicillin 100 U/ml, streptomycin 100 mg/ml, amphotericin B 250 mg/ml (Sigma), and 30% human serum (Irvine Scientific, Santa Ana, CA). The resulting cell cultures were free of contaminating fibroblasts as assessed by morphologic and immunologic criteria. Experiments were conducted with EC at passages 2-6.

ELISA for AECA

The sera were screened for the presence of AECA using ELISA. HDMEC were plated in 96-well plates and allowed to grow to confluence over 24 h. A total of 100 μl of sera diluted 1 : 200 in Hanks balanced salt solution (HBSS) with divalent cations (Irvine) and 1% bovine serum albumin (Sigma) was added to each well and the plates were incubated at $37^{\circ}C$ for 1h. After washing, 100 μl of peroxidase-conjugated goat anti-human IgG (Sigma) or IgM (Sigma) diluted 1 : 1,000 by HBSS with divalent cations and 5% newborn calf serum (Gibco), was added to each well and plates were incubated for 1 h. The plates were again washed and the binding of antibody was quantitated colorimetrically by the addition of tetramethylbenzidine (TMB, 1 mg/ml, Sigma). One ml of a 100 mg/ml stock solution of TMB in acetone was added to 100 ml of distilled water. Ten microliters of 30% H_2O_2 was added immediately prior to use. The chromogenic reaction was stopped with 25 μl 8 N H_2SO_4 and the plates were read spectrophotometrically at 450 nm on an ELISA reader (Dynatech Laboratories Inc., Alexandria, VA, U.S.A.). All data points were done in triplicate.

Sera samples from negative healthy controls, AECA-negative BD patients, AECA-positive BD patients, and sera samples depleted AECA from sera of AECA-positive BD patients using gel chromatography were used in ELISA and flow cytometry for the expression of adhesion molecules.

ELISA for detection of adhesion molecule expression

HDMEC were plated in 96-well flat-bottomed microtiter plates and allowed to grow to confluence over 24 h at a concentration of 4×10^{4} cells per well. HDMEC were incubated with AECA-positive sera of BD patients. A total of 100 μl of either Anti-ICAM-1 antibody (84H10, Immunotech Inc., Westbrook, ME, U.S.A.), anti-VCAM-1 antibody (51-10C9, Pharmingen, San Diego, CA, U.S.A.), or anti-E-selectin antibody (1.2B6, Immunotech Inc., Westbrook, ME, U.S.A.) was added to each well and the plates were incubated at $37^{\circ}C$ for 1 h. After washing, 100 μl of peroxidase-conjugated goat anti-mouse IgG (Sigma), diluted 1 : 500, was added to each well and the plates were incubated for 1 h. The plates

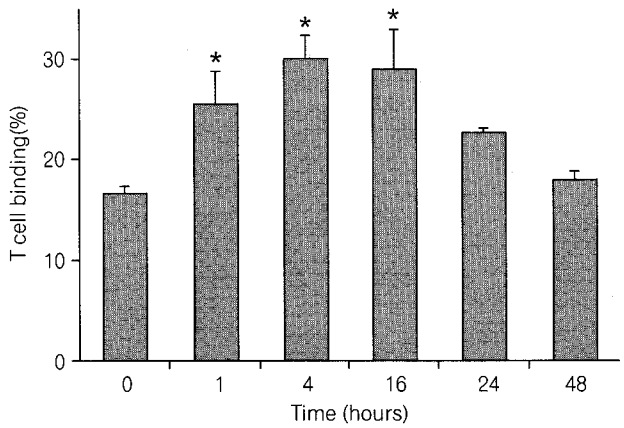

*Fig. 6. Time course of AECA-positive sera of BD patients binding of T lymphocyte to HDMEC monolayers. HDMEC were stimulated with AECA-positive sera of BD patients for 1, 4, 16, 24 and 48 h and then co-incubated with Cr^{51}-labeled T lymphocytes. Data are presented as mean±SD. *p<0.05 vs. untreated HDMEC.*

lymphocyte to HDMEC significantly increased at 1 h, peaked at 4 h and then slowly decreased to a near basal level at 48 h ($p<0.05$).

DISCUSSION

In this study we have analyzed the effects of AECA-positive sera of BD patients on the capability on stimulating HDMEC to lead to an increase in the expression of EC adhesion molecules on the cell surface and to promote the adherence of T lymphocytes. Pretreatment of human endothelial cells *in vitro* with BD patients' sera containing IgM-AECA led to an increase in the expression of ICAM-1, VCAM-1 and E-selectin molecules, and consequently led to an increase in the adhesion of T lymphocytes. This effect was not found with BD patients' sera that contained no detectable AECA.

Although the pathogenesis of BD still remains obscure, autoantibodies to mucosal cells, the presence of circulating immune complex, changes in the amount of immunoglobulin in serum, and antibody and complement deposition on immunofluorescence all show an autoimmune-mediated pathogenesis of BD.[18-20] Lindquist and Osterland first reported AECA in various inflammatory diseases by indirect immunofluoresence study using a mouse kidney.[21] Lee et al. previously reported 49 (37.4%) out of 131 BD patients had IgM AECA but the antibodies did not increase in normal controls or in other disease control groups.[10] We observed circulating antibodies from sera of BD patients to surface antigens on cultured HDMEC by ELISA. This antibody was not detected in healthy controls.

EC are known targets of BRM. Gamble et al. reported that pretreatment with BRM such as interleukin-1 α (IL-1 α), tumor necrosis factor-α (TNF α), lipopolysaccharide and interferon-γ (IFN-γ) led to an increase or induced the expression of adhesion molecules.[22] Furthermore, activated endothelial cells produced leukocyte chemoattractants and costimulatory signals for lymphocyte activation, and thus initiated or amplified inflammatory injury.[23,24] The expression of ICAM-1 and VCAM-1 on HDMEC can be increased by BRM with the onset of increase at 4 to 6 h and reaching a maximum level at 16 to 24 h.[17] In our study, the expression of ICAM-1 and VCAM-1 started to increase at 1h and reached a peak at 16 h and returned to its base level at 48 h. The expression of E-selectin is rapid with a transient peak at 4 to 8 h after stimulation with BRM and disappears after 24 h.[16] In this study, the induction of E-selectin molecules reached a peak at 4 h and then quickly disappeared. These results showed that stimulation of HDMEC by AECA-positive sera of BD patients caused an increase in the expression of EC adhesion molecules in a similar pattern to stimulation with IL-1 α or TNF α. This interesting effect indicated a direct or indirect action of AECA of BD patients on the regulation of the expression of endothelial cell adhesion molecules.

Bevilacqua et al. reported an increase in T lymphocyte adhesion on HUVEC after stimulation with IL-1 α, TNF α, lipopolysaccharide or IFN-γ.[25] Thornhill et al. reported a similar study on T lymphocyte adhesion and observed an increase in adhesion at 4 h continuing to 72 h after stimulation with BRM.[26] In our study, we observed an increase in the adhesion of T lymphocytes after stimulation of HDMEC with AECA-positive sera of BD patients and found it to be time dependent with an increase in adhesion starting at 1h and continuing until 48 h. We also found that the T lymphocyte adhesion pattern was similar following stimulation with IL-1 α or TNF α.

A recent report by Carvalho et al. showed that an increased adhesion of U937 cells was accompanied by increased expression of endothelial ICAM-1, VCAM-1 and E-selectin molecules.[27] Calvalho et al. suggested that AECA may activate EC by an autocrine or paracrine action of IL-1 α.[27] It can be assumed in our study that AECA acted on HDMEC to induce cytokines such as IL-1 α or TNF α which then activated