

## Human $\alpha$ -Enolase From Endothelial Cells as a Target Antigen of Anti-Endothelial Cell Antibody in Behçet's Disease

Kwang Hoon Lee,<sup>1</sup> Hae-Shin Chung,<sup>2</sup> Hyoung Sup Kim,<sup>2</sup> Sang-Ho Oh,<sup>2</sup> Moon-Kyung Ha,<sup>3</sup> Ja-Hyun Baik,<sup>4</sup> Sungnack Lee,<sup>5</sup> and Dongsik Bang<sup>1</sup>

**Objective.** To identify and recombine a protein of the human dermal microvascular endothelial cell (HDMEC) that specifically reacts with anti-endothelial cell antibody (AECA) in the serum of patients with Behçet's disease (BD), and to evaluate the usefulness of this protein in BD.

**Methods.** The proteomics technique, with 2-dimensional gel electrophoresis and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry, was used to identify and recombine HDMEC antigen. Western blotting and enzyme-linked immunosorbent assay (ELISA) of recombinant protein isolated by gene cloning were performed on serum from healthy controls, patients with BD, and patients with other rheumatic diseases (rheumatoid arthritis, systemic lupus erythematosus, and Wegener's granulomatosis).

**Results.** Eighteen of 40 BD patients had serum IgM antibody to HDMEC antigen. The purified protein that reacted with AECA in BD patient sera was found to be  $\alpha$ -enolase by 2-dimensional gel electrophoresis followed by immunoblotting and MALDI-TOF mass spectrometry. Recombinant  $\alpha$ -enolase protein was isolated

and refined by gene cloning. On Western blots, AECA-positive IgM from the sera of patients with active BD reacted strongly with recombinant human  $\alpha$ -enolase. BD patient sera positive for anti- $\alpha$ -enolase did not react with human  $\gamma$ -enolase. On dot-blotting, reactivity to human  $\alpha$ -enolase was detected only in the IgM-positive group. Fifteen of the 18 AECA-positive sera that were positive for the HDMEC antigen showed reactivity to recombinant  $\alpha$ -enolase IgM antibody by ELISA.

**Conclusion.** The  $\alpha$ -enolase protein is the target protein of serum AECA in BD patients. This is the first report of the presence of IgM antibodies to  $\alpha$ -enolase in endothelial cells from the serum of BD patients. Although further studies relating this protein to the pathogenesis of BD will be necessary,  $\alpha$ -enolase and its antibody may prove useful in the development of new diagnostic and treatment modalities in BD.

Behçet's disease (BD) is a chronic, multisystem disorder characterized by a recurrent inflammatory reaction. Serious complications, such as blindness or intestinal perforations, can occur. Because of the lack of definitive diagnostic tests for BD, the diagnosis is based solely on the clinical manifestations. The pathogenesis of BD remains obscure. Clinically, BD patients experience recurrent thrombophlebitis, thrombosis, and cutaneous vasculitis (1). Histopathologic changes consisting of perivascular mononuclear cellular infiltrates, endothelial cell swelling or necrosis, partial obliteration of the vessel lumen, and occasional fibrinoid necrosis of vessels have been observed. In more severe forms, small to medium-sized arteries, veins, and capillaries may be affected by a necrotizing or granulomatous vasculitis (2,3).

Anti-endothelial cell antibody (AECA) was first detected in various inflammatory diseases by indirect immunofluorescence using a mouse kidney substrate (4). AECA has been demonstrated in the serum of patients

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<sup>1</sup>Kwang Hoon Lee, MD, PhD, Dongsik Bang, MD, PhD: Cutaneous Biology Research Institute, and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea; <sup>2</sup>Hae-Shin Chung, MD, PhD, Hyoung Sup Kim, MD, Sang-Ho Oh, MD: Yonsei University College of Medicine, Seoul, Korea; <sup>3</sup>Moon-Kyung Ha, MS: Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea; <sup>4</sup>Ja-Hyun Baik, PhD: Medical Research Center, Yonsei University College of Medicine, Seoul, South Korea; <sup>5</sup>Sungnack Lee, MD, PhD: Ajou University School of Medicine, Suwon, South Korea.

Address correspondence and reprint requests to Dongsik Bang, MD, PhD, Department of Dermatology, Yonsei University College of Medicine, CPO Box 8044, Seoul, South Korea. E-mail: dbang@yumc.yonsei.ac.kr.

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with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), mixed connective tissue disease, scleroderma, and dermatomyositis, as well as patients with Wegener's granulomatosis (WG), primary retinal vasculitis, Kawasaki disease, acute hemolytic uremic syndrome, diabetes mellitus, organ transplants, hypoparathyroidism, multiple sclerosis, thrombotic thrombocytopenic purpura, angioedema, and Buerger's disease (5,6). AECA has also been demonstrated in the serum of BD patients (7–10) and has been associated with disease activity and vasculitis symptoms (6,7).

Even though a large number of molecules on the surface of endothelial cells have been characterized, only a few reports have studied the characteristics of the antigen recognized by AECA. In a previous study (9), we used an enzyme-linked immunosorbent assay (ELISA) to detect circulating antibodies against human dermal microvascular endothelial cells (HDMECs) in the serum of patients with BD. Serum IgM antibody against HDMEC was detected in 49 of 131 BD patients. On Western blots, IgM-positive BD serum reacted with the 44–50-kd HDMEC surface antigen, whereas IgM-positive SLE serum reacted with the 81-kd HDMEC surface antigen (9).

Proteomics is a field of science that evaluates a large number of proteins expressed from a given cell line or organism. The technology of proteomics has been used as a method of discovering the target protein specific to a particular disease by searching for the expression or modification of the protein (11,12). To characterize the AECA-binding HDMEC antigen, which is closely related to the pathogenesis of BD, we first identified a target protein by 2-dimensional (2-D) gel electrophoresis and immunoblotting, and then searched for a similar protein after the amino acids were sequenced by mass spectrometry. We next searched for the DNA sequence of the target protein at the National Center for Biotechnology Information (NCBI) and purified the recombinant target protein by gene cloning. We then investigated the reactivities of the recombinant target protein in BD.

## PATIENTS AND METHODS

**Patients.** This study included 40 BD patients who fulfilled the diagnostic criteria of the International Study Group for BD (13). All patients had active disease, meeting at least 2 major criteria with or without any number of minor criteria. Sera from 40 healthy volunteer donors, 6 patients with RA, 5 patients with SLE, and 2 patients with WG were used as controls. We measured rheumatoid factor (RF), antinuclear antibody (ANA), and antineutrophil cytoplasmic antibody (ANCA) in all serum samples from BD patients.

**Isolation and culture of HDMECs.** HDMECs were isolated from human newborn foreskins by a previously reported technique (14). Briefly, the tissue was cut into 5-mm<sup>2</sup> sections. The sections were incubated for 40 minutes at 37°C in trypsin solution and then washed 3 times in phosphate buffered saline (PBS). Pressure was initially applied to the center of each tissue section, and the released cells were pushed outward, toward the periphery of the tissue section and into the medium. The primary isolated cells were collected by centrifugation (800g for 1 minute) and resuspended in endothelial basal media (Clonetics, San Diego, CA), with 5 ng/ml of epidermal growth factor (Clonetics), 1 µg/ml of hydrocortisone acetate (Sigma, St. Louis, MO),  $5 \times 10^{-5}M$  dibutyryl cAMP (Sigma), 100 units/ml of penicillin, 100 µg/ml of streptomycin, 250 µg/ml of amphotericin B (Sigma), and 30% human serum (Irvine Scientific, Santa Ana, CA).

The resultant cell cultures were free of contaminating fibroblasts, as assessed by morphologic and immunologic criteria. Until required for experiments, cells from the second passage were cultured in endothelial basal medium (Clonetics) containing 5 µg of human epidermal growth factor, 1 µg/ml of hydrocortisone acetate,  $5 \times 10^{-5}M$  dibutyryl cAMP, 100 units/ml of penicillin, 100 µg/ml of gentamicin, 250 µg/ml of amphotericin B, and 2% fetal bovine serum.

**ELISA.** HDMECs were plated in microtiter plates and allowed to grow to confluence over 24 hours. The plates were washed 3 times with 0.05% PBS-Tween 20 (PBST) to block nonspecific binding. Then, 100 µl of sera from normal controls and BD patients, diluted 1:50 in Hanks' balanced salt solution (HBSS) with divalent cations (Irvine Scientific) and 1% bovine serum albumin (BSA; Sigma), was added to each well of unfixed HDMEC, and the plates were incubated for 1 hour at 37°C. Peroxidase-conjugated goat anti-human IgG or IgM diluted 1:1,000 in HBSS with divalent cations and 1% BSA was added to each well, and the plates were incubated for 1 hour at 37°C. Three wells containing diluent only were used as blanks to provide a background level.

Antibody binding was quantified colorimetrically by adding tetramethylbenzidine (Sigma) as substrate. One microliter of 30% H<sub>2</sub>O<sub>2</sub> was added immediately prior to use. The chromogenic reaction was stopped with 8N H<sub>2</sub>SO<sub>4</sub>, and the plates were read spectrophotometrically at 450 nm on an ELISA reader (Dynatech, Alexandria, VA). Positivity was defined as an optical density (OD) greater than the mean plus 3 SD for the 40 normal control sera.

**Gel electrophoresis and immunoblotting of HDMEC antigen.** HDMEC antigen in EDTA/BSA was dissolved in buffer (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1% Triton X-100) on ice for 30 minutes. After centrifugation at 10,000g for 30 minutes at 4°C, the supernatant was collected and mixed with the same amount of sample buffer. Samples were then loaded into the wells of a 10% polyacrylamide gel, and electrophoresis was performed at a fixed current for 45–90 minutes. The separated gel was transferred to a nitrocellulose membrane, washed with 0.05% PBST, and incubated overnight at 4°C in blocking buffer. After a second overnight incubation at 4°C (with gentle agitation) in sera from normal controls and BD patients, diluted 1:50 with primary antibody dilution buffer, the membrane was washed 3 times with PBST and then incubated with peroxidase-conjugated goat anti-human IgM for 2 hours. After washing with PBST, diaminobenzidine with

30%  $H_2O_2$  was added, and the membrane was incubated at 37°C for 10 minutes.

**Fractionation of IgM from the AECA-positive BD patient sera and confocal laser microscopy.** The column was filled with PBS treated with Ultragel AcA 34 (Sigma). Serum was passed through the column, and 4 fractions were collected. Reaction of the isolated IgM antibody against HDMEC antigen was confirmed by gel electrophoresis and immunoblotting as described above. Cultured HDMECs were trypsinized and replated in 8-well chamber slides (Becton Dickinson, Mountain View, CA). The fractionated IgM obtained from the sera of normal controls and AECA-positive BD patients (diluted 1:20 in HBSS with divalent cations and 1% BSA) was added to unfixed HDMEC monolayers and incubated with fluorescein isothiocyanate-conjugated goat anti-human IgM. The slides were washed and evaluated for fluorescence using a confocal laser microscope (TCSNT; Leica, Heidelberg, Germany).

**Protein preparation.** Cells were washed twice with ice-cold PBS and harvested using homemade scrapers to prepare the supernatant. The resulting pellets were resuspended in 500  $\mu$ l of solubilization buffer consisting of 7M urea, 2M thiourea, 4% CHAPS, 2 mM tributylphosphosine (TBP), 0.5% carrier ampholytes, 40 mM Tris, and 0.001% bromphenol blue. Samples were mixed on a vortex mixer for 30 seconds and ultrasonicated using an Ultrasonic Processor XL (XL2020; Misonix, New York, NY); this sonication procedure was repeated 4 times. Nucleic acids were digested with endonuclease (Sigma) using 150 units/ml of sample for 30 minutes at room temperature. Samples were centrifuged at 10,000g for 10 minutes and supernatants were collected. Extra protein extracts were stored at -80°C until used.

**Two-dimensional gel electrophoresis.** Rehydration of supernatants was performed using an 18-cm, pH 3–10, linear immobilized pH gradient (IPG) strip (Amersham Pharmacia Biotech, Piscataway, NJ) for 24 hours. Isoelectric focusing (IEF) was performed with rehydrated IPG strips using a Protean IEF cell (Bio-Rad, Hercules, CA) for the appropriate time. After IEF, the IPG strips were equilibrated for 25 minutes in buffer consisting of 6M urea, 2% sodium dodecyl sulfate (SDS), 5M Tris, 20% glycerol, 25% acrylamide, and 200 mM TBP.

To make the polyacrylamide gel, a 9% buffer solution containing 1.875M Tris buffer, 40% stock acrylamide, distilled water, 16% buffer solution, 40% stock acrylamide, and 50% glycerol was mixed, and a 9–16% gradient polyacrylamide gel was prepared. After adding agarose buffer, which included 0.5% agarose and 0.001% bromphenol blue dye, the IPG strip was embedded, and cathode-running buffer (24.8 mM Tris, 192 mM glycine, and 0.1% SDS) was added. Gels were run initially at 3 mA/gel for 2 hours and then at 18 mA/gel for ~16 hours.

**Two-dimensional immunoblotting and isolation of protein spots.** The gel was transferred to a nitrocellulose membrane by semidry transfer (Bio-Rad) at 500 mA for 2 hours. Immunoblotting was performed with isolated AECA IgM antibody from the serum of BD patients. The protein spot was separated with a clean razor blade and dehydrated in 50 mM ammonium bicarbonate solution for 5 minutes. After dehydration in acetonitrile in a speedback evaporator, the protein had dried sufficiently. After treatment in 25 mM ammonium bicarbonate solution containing 0.1  $\mu$ g of trypsin at 37°C for 16 hours, tryptic peptides were eluted several times and then thoroughly dried in a speedback evaporator.

**Peptide fingerprint by mass spectrometry.** Samples mixed with saturated  $\alpha$ -cyano-4-hydroxycinnamic acid were dropped onto a matrix-assisted laser desorption ionization (MALDI) plate and dried at room temperature. The mass spectrum was obtained by averaging 40–50 individual laser shots, using a MALDI Reflectron time of flight instrument (Micromass, Manchester, UK). The autolytic peptide peak ( $m/z$  842.50 and 2211.10) of bovine trypsin was used as an internal standard.

**Scanning the data for a peptide fingerprint.** We entered our protein mass data into the MS-Fit program, a peptide mass fingerprinting tool provided by the University of California, San Francisco (UCSF) (available at <http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>), which uses an NCBI scanning algorithm, to search for the identity of the protein. The error limit was within 50 parts per million.

**Synthesis of complementary DNA (cDNA) by reverse transcription-polymerase chain reaction (RT-PCR).** Whole RNA was isolated from cultured HDMECs using TRIzol reagent, based on the acid guanidinium thiocyanate-phenol-chloroform extraction method according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNA was synthesized from 10  $\mu$ g of total RNA using the Superscript II preamplification system (Invitrogen Life Technologies). Whole RNA was mixed with random hexamer (Promega, Madison, WI), dNTPs, buffer, dithiothreitol, and RNase to proper density. After adding Superscript II reverse transcriptase, samples were incubated at 42°C for 1 hour. Synthesized first-strand cDNA was then mixed with dNTPs,  $MgCl_2$ , and buffer. DNA polymerase and primer corresponding to the protein were mixed. Double-stranded cDNA was synthesized by 40 cycles of denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes.

Primers were designed according to the coding sequences for human  $\alpha$ -enolase, and *Bam* HI and *Xho* I restriction sites were placed at their respective terminal ends to facilitate subcloning of the PCR product into the expression vector pGEX-4T-1. Primer sequences were 5'-CAGTTGGATCC-TCTATTCTCAAGATCCATGCCA-3' for the sense primer and 5'-ATCCTCGAGTTACTTGGCCAAGGGRTTTTCTG-AAGTTCCTG-3' for the antisense primer.

**Subcloning of cDNA.** Amplification products were separated on agarose gels, and the band of the desired size was excised and purified using 6N NaI and GlassMilk (Invitrogen, Carlsbad, CA). The GlassMilk was precipitated by centrifugation at 1,000 revolutions per minute for 1 minute. After washing twice with washing buffer, distilled water was added, and the sample was incubated at 50°C for 10 minutes. The sample was then centrifuged at 1,000 rpm for 1 minute, and DNA was extracted from the supernatants. Both pGEX-4T-1 vector DNA and cloned cDNA were cleaved by *Bam* HI and *Xho* I and ligated with T4 ligase. Recombinant plasmid DNA was introduced into *Escherichia coli* DH5 $\alpha$  cells. Transformed *E coli* DH5 $\alpha$  cells were cultured in Luria-Bertani (LB) agar supplemented with ampicillin for 16 hours. The single colony on the LB agar was cultured in LB broth for 12 hours. After miniprep preparation using the Wizard Plus SV Miniprep DNA purification system (Promega), followed by treatment with the restriction enzymes *Bam* HI and *Xho* I, we confirmed whether the cloned cDNA was inserted into the vector.

**Table 1.** Frequency of AECA to HDMEC antigen in sera from normal subjects and patients with Behçet's disease\*

Group	% AECA positive		
	IgG only	IgM only	IgG + IgM
Normal subjects (n = 40)	0 (0)	0 (0)	0 (0)
Behçet's disease patients (n = 40)	4 (10.0)	15 (37.5)	3 (7.5)

\* AECA = anti-endothelial cell antibody; HDMEC = human dermal microvascular endothelial cell.

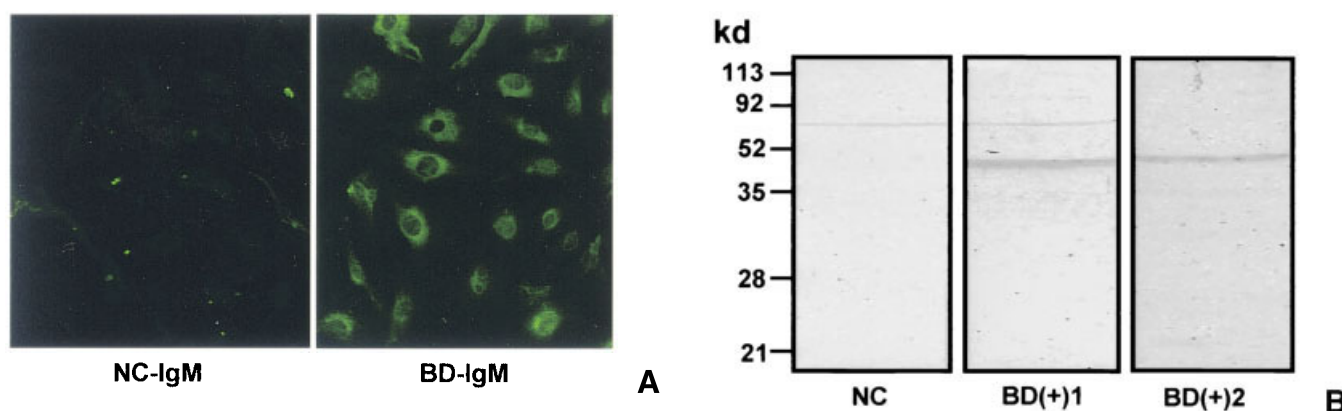
**Gene expression and purification of recombinant protein.** Transformed *E coli* was cultured in LB broth until the absorption density at 600 nm reached 0.45–0.55, as measured by colorimetry. To induce the expression of recombinant protein, 1 mM IPTG was added and incubation was continued for an additional 16 hours at 25°C. After centrifugation, PBS with proteinase, in an amount up to 1/100 of the cultured volume, was added. Cells were lysed by sonication on ice. Triton X-100 was then added to a final concentration of 1%, and the suspension was mixed gently for 30 minutes to facilitate solubilization of the proteins.

Supernatant containing the desired protein fused with glutathione S-transferase (GST) was extracted by centrifugation at 13,000 rpm for 10 minutes at 4°C. Glutathione-Sepharose 4B beads were added, and GST fusion protein was adsorbed onto the beads by shaking incubation for 12 hours. After the beads were isolated by centrifugation, they were washed with PBS and with PBS containing proteinase. The protein adsorbed into the beads was isolated by incubation with elution buffer at room temperature for 30 minutes. Each 50  $\mu$ l of the GST-human  $\alpha$ -enolase fusion protein, which was diluted to a concentration of 2  $\mu$ g/ml with carbonate buffer (pH 9.6), was plated in the microtiter plates and allowed to stand at 4°C for 18 hours. Glutathione-Sepharose 4B beads were treated with thrombin protease. Only the required human  $\alpha$ -enolase was isolated from the GST fusion protein that had been adsorbed on the beads. We confirmed the purity of this protein by SDS-polyacrylamide gel electrophoresis (PAGE).

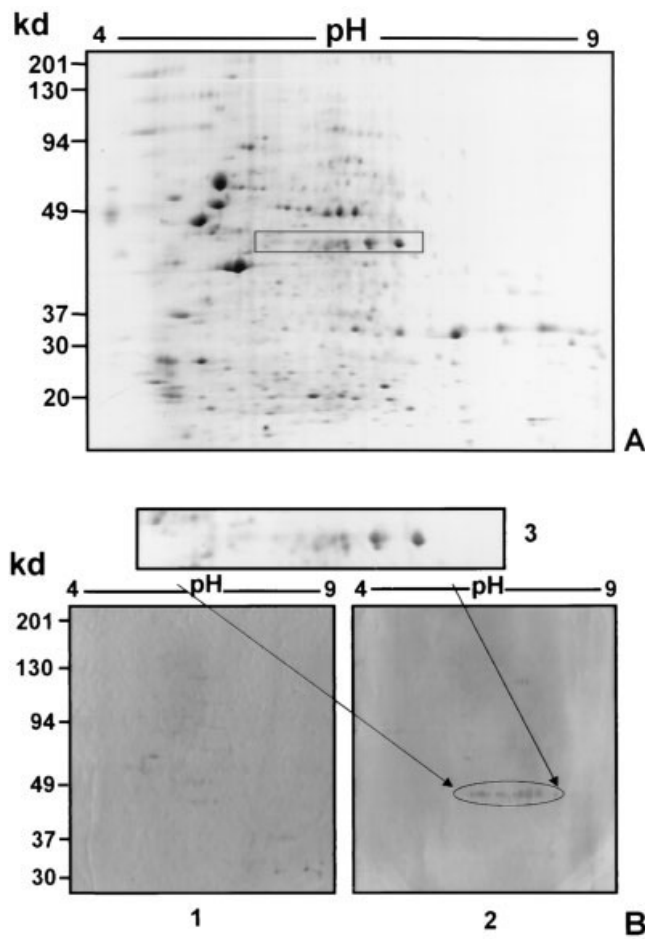
**Identification of recombinant protein by gel electrophoresis and immunoblotting.** After mixing human  $\gamma$ -enolase (Polysciences, Warrington, PA) and the purified recombinant human  $\alpha$ -enolase with the same amount of sample buffer, the samples were loaded into the wells of a 10% polyacrylamide gel and subjected to electrophoresis at a fixed electric current for 45–90 minutes. The separated gel was transferred to a nitrocellulose membrane, and the membrane was washed with PBST and incubated overnight at 4°C in blocking buffer. The membrane was then incubated overnight at 4°C (with gentle agitation) with IgM derived from the sera of normal controls, BD patients, and other rheumatic disease patients, diluted 1:50 with primary antibody dilution buffer. Goat anti-human  $\alpha$ -enolase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control at a dilution of 1:100. After washing 3 times with PBST, the membrane was incubated at room temperature for 2 hours with peroxidase-conjugated goat anti-human IgM. Diaminobenzidine containing 30% H<sub>2</sub>O<sub>2</sub> was then added, and the membrane was incubated at 37°C for 10 minutes.

Dot-blotting was performed to identify the subclass of the immunoglobulin that on Western blotting appeared to react with human  $\alpha$ -enolase. Each 3  $\mu$ l of recombinant  $\alpha$ -enolase, which was diluted to a concentration of 7  $\mu$ g/ $\mu$ l with carbonate buffer (pH 9.6) was dropped onto a nitrocellulose membrane, the membrane was washed with PBST, and then incubated overnight at 4°C in blocking buffer. Blots were cut from the membrane and incubated with IgM or IgG from the sera of BD patients, diluted 1:50 with primary antibody dilution buffer, and gently agitated overnight at 4°C. After washing 3 times with PBST, the membrane was incubated with peroxidase-conjugated goat anti-human IgM or IgG for 2 hours. Diaminobenzidine containing 30% H<sub>2</sub>O<sub>2</sub> was added, and the membrane was incubated at 37°C for 10 minutes.

**Detection of anti-human  $\alpha$ -enolase antibodies in BD sera.** An IgM ELISA was performed as described above to examine the reactivity of purified  $\alpha$ -enolase with sera from the 40 patients with BD and the 13 patients with other rheumatic diseases.



**Figure 1.** **A,** Immunofluorescence labeling of unfixed HDMECs was performed using IgM from the serum of a normal control subject and an AECA-positive BD patient. **B,** Western blot analysis of 2 different Behçet's disease (BD) patient sera and a normal control (NC) serum, using human dermal microvascular endothelial cells (HDMECs) as substrate. Western blotting showed no specific positive bands in the sera of healthy subjects, but showed a band between 44 kd and 50 kd of the HDMEC protein in the anti-endothelial cell antibody (AECA)-positive sera of BD patients.



**Figure 2.** A, Two-dimensional (2-D) gel electrophoresis of cultured HDMECs after isoelectric focusing on an 18-cm, pH 3–10, linear immobilized pH gradient strip, showing ~2,000 protein spots. Boxed area shows protein spots in the range of 44–50 kd. B, On 2-D immunoblotting, 3–4 protein spots in the range of 44–50 kd reacted with IgM from an AECA-positive BD patient serum. Inset, Higher magnification of circled area in panel 2. Panel 1 shows serum from a normal control subject. Results are representative of 3 separate experiments using 3 different serum samples. See Figure 1 for other abbreviations.

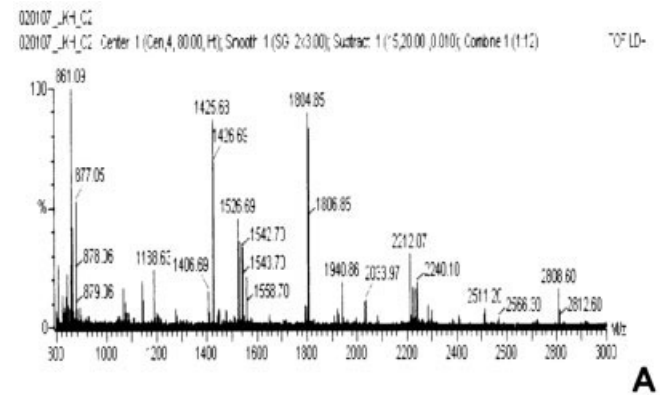
**RESULTS**

**Frequency of antibody against HDMEC antigen in sera from patients with BD.** In the IgM ELISA for HDMEC antigen, the mean ( $\pm$ SD) OD of sera from normal control subjects (n = 40) was  $0.084 \pm 0.031$ . Values higher than 0.177, which is the sum of the mean OD for normal controls and 3 times the SD, were considered positive. Eighteen of the 40 BD patients (45%) had positive results: 15 were positive for IgM only, and 3 were positive for both IgM and IgG. IgM antibody was not detected in the other BD patients (Table 1). The mean OD of sera from the patients with BD was  $0.0278 \pm 0.106$ .

In the IgG ELISA, the mean OD of sera from normal control subjects (n = 40) was  $0.091 \pm 0.052$ , and the mean OD of sera from the 40 BD patients was  $0.142 \pm 0.176$ . Seven of the 40 BD patients (17.5%) had positive results: 4 were positive for IgG only, and 3 were positive for both IgG and IgM. All sera from the BD patients were negative for RF, ANA, and ANCA.

**Reactivity of circulating antibody to HDMEC on Western blotting.** Fractionated IgM obtained from the sera of AECA-positive BD patients was found to react with surface antigen on unfixed HDMECs, by confocal laser microscopy. These findings were compared with those in IgM obtained from the sera of healthy control subjects (Figure 1A).

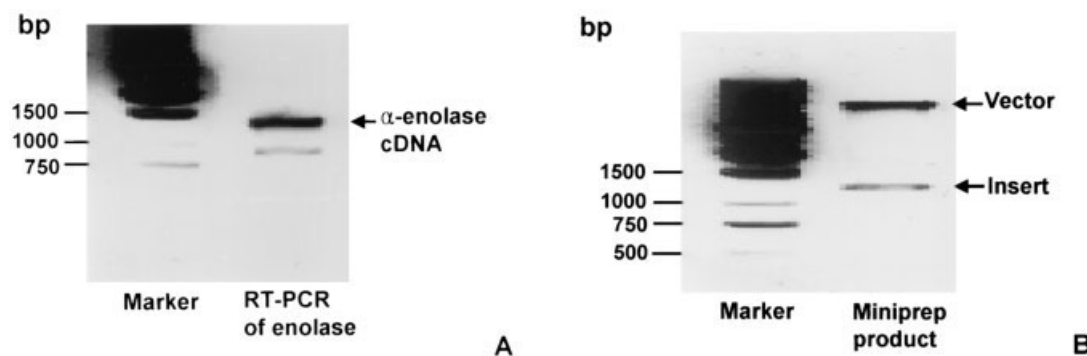
To identify the protein related to the AECA positivity in BD patient sera, we performed gel electro-



Name: alpha-enolase MW: 47169.4 Da pI: 7.0 Sequence coverage 41%.

Masses Submitted	MH+ Matched	$\Delta$ ppm	Peptide sequence numbers	Proposed peptide sequence	Modifications
1406.747	1406.717	22	16–28	(R) GNPTVEVDLFTSK (G)	
1425.768	1425.727	29	270–281	(R) YISPDQLADLYK (S)	
1540.820	1540.783	24	240–253	(K) VVIGMDVAASEFFR (S)	
1556.814	1556.778	23	240–253	(K) VVIGMDVAASEFFR (S)	1Met-ox
1804.978	1804.945	19	33–50	(R) AAVPSGASTGIYEALER (D)	
1908.006	1907.988	9.7	163–179	(K) LAMQEFMILPVGAAANFR (E)	
2033.082	2033.056	13	307–326	(K) FTASAGIQVGGDDLTVTNPK (R)	

**Figure 3.** A, Peptide fingerprint obtained by mass spectrometry of HDMEC protein spots that showed a strong positive reaction to AECA on 2-dimensional immunoblotting. The peaks shown on the peptide fingerprint correspond to  $\alpha$ -enolase. TOF = time of flight; LD = laser desorption. B, Analysis of our protein mass data using the MS-Fit program (see Patients and Methods for details) showed that the protein spots possessed 41% identity with the amino acid sequences of  $\alpha$ -enolase having a molecular weight of 47 kd and an isoelectric point of 7.0. MH+ = peptide plus H+. See Figure 1 for other definitions.



**Figure 4.** **A**, Results of reverse transcription–polymerase chain reaction (RT-PCR) of human dermal microvascular endothelial cell (HDMEC)  $\alpha$ -enolase. Total RNA of HDMEC was isolated, and primers were designed based on the coding sequence for human  $\alpha$ -enolase. *Bam* HI and *Xho* I restriction sites were placed at the respective primer terminals to facilitate subcloning of the PCR product into the pGEX-4T-1 expression vector. **B**, Both pGEX-4T-1 vector DNA and  $\alpha$ -enolase cDNA were cleaved by *Bam* HI and *Xho* I and then ligated with T4 ligase. Recombinant plasmid DNA was introduced into *Escherichia coli* DH5 $\alpha$  cells. To confirm the introduction of the recombinant plasmid DNA into *E. coli*, we performed a miniprep. We confirmed the vector (4,969 bp) and inserted double-stranded DNA (1,305 bp) within the plasmid by treating recombinant plasmid DNA with the restriction enzymes *Bam* HI and *Xho* I.

phoresis with cultured HDMECs and Western blotting of sera from normal subjects and BD patients who had positive results on the IgM ELISA. Western blotting showed no specific positive bands in the sera of normal control subjects, but a band of HDMEC protein between 44 kD and 50 kD was identified in AECA-positive sera from patients with BD (Figure 1B).

**Isolation and identification of the specific antigen reacting with AECA, using the proteomics method.** About 2,000 protein spots were observed on 2-D gel electrophoresis of cultured HDMECs after isoelectric focusing on the 18-cm, pH 3–10, linear IPG strip (Figure 2A). Two-dimensional immunoblotting of IgM obtained from the sera of AECA-positive BD patients showed 3–4 protein spots of 44–50 kD (Figure 2B). These spots were removed and examined by mass spectrometry, and a peptide fingerprint was obtained (Figure 3A). Using the MS-Fit program provided by UCSF (NCBI scanning algorithm), we found that 7 of 17 peptides coincided with human  $\alpha$ -enolase (within 30 ppm) and that the protein spots showed a 41% sequence homology with the amino acid sequences of  $\alpha$ -enolase (Figure 3B). Although their isoelectric points and molecular weights were a little different, all spots were finally identified as 47-kD human  $\alpha$ -enolase (2-phospho-D-glycerate hydrolase) with an isoelectric point of 7.0.

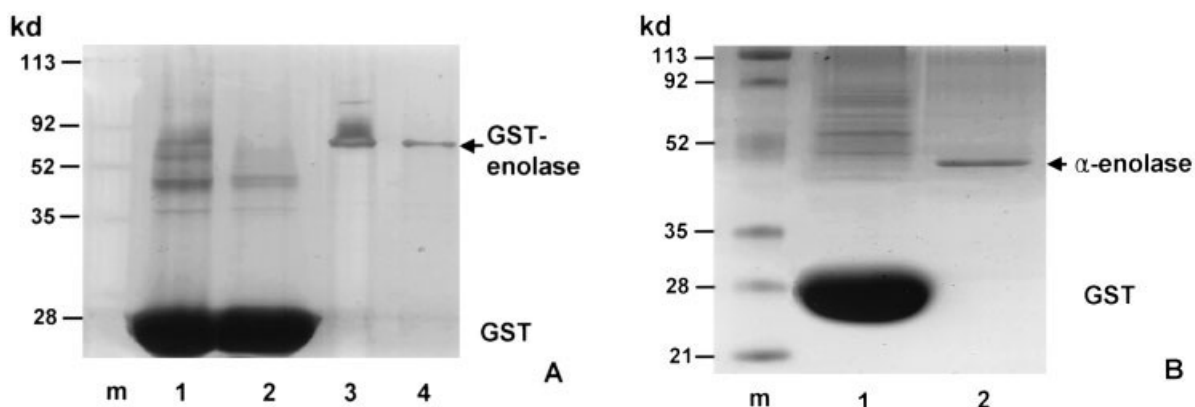
**Gene cloning of  $\alpha$ -enolase in HDMECs.** After isolating whole RNA from cultured HDMECs, we performed RT-PCR on the  $\alpha$ -enolase, using the identified sequence of human  $\alpha$ -enolase from GenBank, and identified the cDNA of  $\alpha$ -enolase at 1.3 kbp (Figure 4A).

The recombinant plasmid DNA was obtained from a miniprep of transformed *E. coli* by subcloning of  $\alpha$ -enolase. We confirmed the vector (4,969 bp) and inserted double-stranded DNA (1,305 bp) into the plasmid (Figure 4B).

**Expression and purification of recombinant  $\alpha$ -enolase.** Protein expression was stimulated with IPTG, and a highly expressed recombinant protein was purified. Following SDS-PAGE, only 1 band of GST-human  $\alpha$ -enolase fusion protein (75 kD) was observed (Figure 5A). After thrombin treatment of the GST fusion protein, SDS-PAGE revealed a single band of 47 kD recombinant human  $\alpha$ -enolase produced by gene cloning (Figure 5B).

**Reactivity of BD sera with recombinant human  $\alpha$ -enolase.** Immunoblotting with recombinant human  $\alpha$ -enolase was performed to examine its reactivity with sera from patients with BD. AECA-positive IgM obtained from the sera of patients with active BD and from the sera of patients with WG reacted strongly with recombinant human  $\alpha$ -enolase (Figure 6A). Sera from normal controls, RA patients, and SLE patients showed negative results. Only 1 BD serum, which was AECA-negative by IgM ELISA, reacted weakly with human  $\alpha$ -enolase (data not shown).

Western blotting was performed to determine the reactivities of BD patient sera to enolase isoforms. BD patient sera that were positive for anti- $\alpha$ -enolase antibody did not react with human  $\gamma$ -enolase (Figure 6B). On dot-blotting, reactivity to human  $\alpha$ -enolase was detected only in the IgM-positive group. No positive



**Figure 5.** **A**, Polyacrylamide gel electrophoresis of purified glutathione S-transferase (GST)–human  $\alpha$ -enolase fusion protein produced by gene cloning. GST (28 kd) obtained from *Escherichia coli* transformed with vector only was used as a control. Lane m, Marker; lane 1, bead after extraction of GST; lane 2, extracted GST; lane 3, bead after extraction of GST-enolase; lane 4, extracted GST-enolase. **B**, Polyacrylamide gel electrophoresis of human  $\alpha$ -enolase produced by treatment with thrombin. After thrombin treatment, a single band of recombinant human  $\alpha$ -enolase was isolated from the GST fusion protein adsorbed on glutathione–Sepharose 4B beads. Lane m, Marker; lane 1, purified GST; lane 2, purified  $\alpha$ -enolase identified at 47 kd after thrombin treatment.

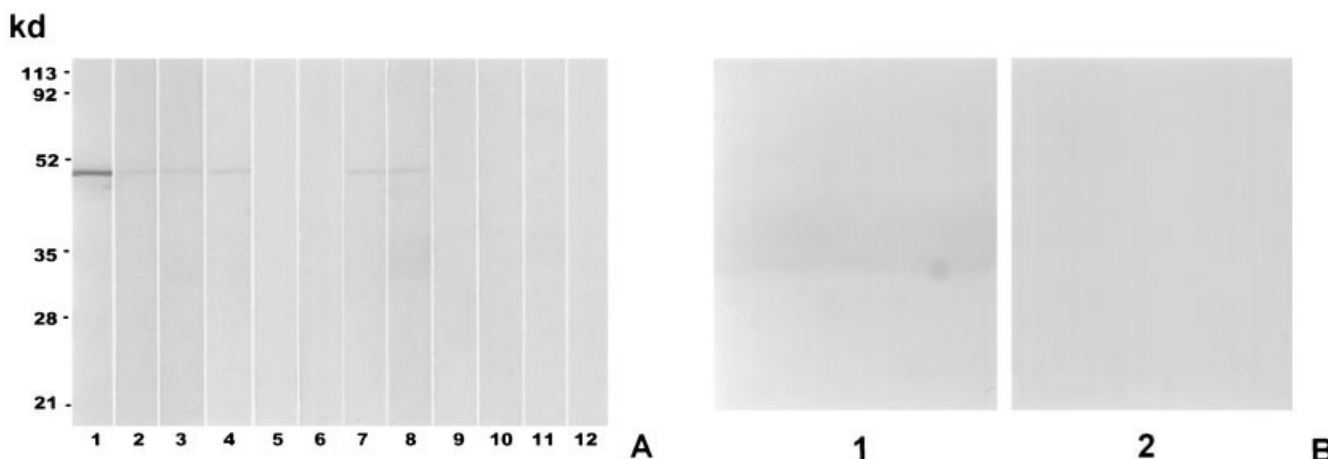
reaction was observed with sera from the IgG-positive group (Figure 7).

Specific positive signals against recombinant human  $\alpha$ -enolase were detected by IgM ELISA of serum samples from 15 of the 40 BD patients (37.5%). Fifteen of the 18 patients (83.3%) whose sera showed positive reactivity to HDMEC antigen in the previous IgM ELISA showed positive reactivity (Table 2). Anti- $\alpha$ -enolase antibodies in sera from patients with other

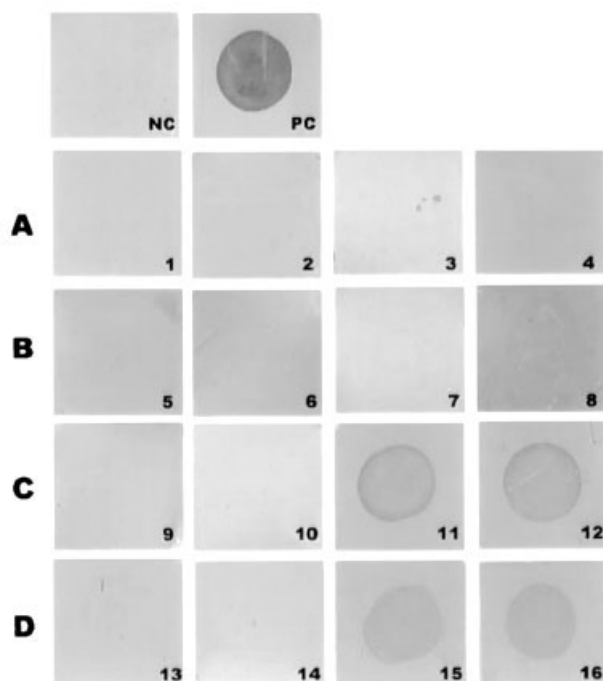
systemic rheumatic diseases (6 with RA, 5 with SLE, and 2 with WG) were also screened by IgM ELISA. One of the 6 RA sera (16.7%) and both of the WG sera (100%) reacted with the human  $\alpha$ -enolase. However, none of the 5 SLE sera showed reactivity.

**DISCUSSION**

Although AECAs have been detected in a variety of inflammatory diseases, there are a few reports of the



**Figure 6.** **A**, Reactivity of serum AECAs from BD patients to purified  $\alpha$ -enolase on immunoblotting. AECA-positive IgM from the sera of patients with active BD (lanes 2, 3, and 4) and Wegener’s granulomatosis (lanes 7 and 8) reacted strongly with purified  $\alpha$ -enolase on immunoblotting. Normal control sera (lanes 5 and 6), rheumatoid arthritis patient sera (lanes 9 and 10), and systemic lupus erythematosus patient sera (lanes 11 and 12) did not react with  $\alpha$ -enolase. Goat anti- $\alpha$ -enolase antibody (lane 1) was used as a positive control. **B**, Immunoreactivities of sera from BD patients against  $\alpha$ -enolase (panel 1) and  $\gamma$ -enolase (panel 2) isoforms. Recombinant human  $\alpha$ -enolase and human  $\gamma$ -enolase were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, transferred onto the membrane, and probed with a BD serum sample containing anti- $\alpha$ -enolase antibodies (1:100 dilution). See Figure 1 for definitions.



**Figure 7.** Dot-blots of human  $\alpha$ -enolase of HDMECs from **Top**, normal control (NC) sera and goat anti-human  $\alpha$ -enolase antibody (positive control [PC]), **A**, IgG-negative, IgM-negative sera from 2 representative BD patients, **B**, IgG-positive, IgM-negative sera from 2 BD patients, **C**, IgG-negative, IgM-positive sera from 2 BD patients, and **D**, IgG-positive, IgM-positive sera from 2 BD patients. Samples were treated with peroxidase-conjugated goat anti-human IgG (panels 1, 2, 5, 6, 9, 10, 13, and 14) or IgM (panels 3, 4, 7, 8, 11, 12, 15, and 16), and then tested by enzyme-linked immunosorbent assay for IgG or IgM antibody reactivity to HDMEC antigen. See Figure 1 for other definitions.

association of AECA with the clinical manifestations of vasculitis. It has been shown that when vasculitis symptoms improve in patients with RA, AECA titers tend to decrease (15). Moreover, high titers of AECA in pa-

**Table 2.** Frequency of autoantibodies to recombinant human  $\alpha$ -enolase purified from cultured human dermal microvascular endothelial cells obtained from normal subjects, patients with Behçet's disease, and patients with other rheumatic diseases

Group	No. of subjects	No. (%) positive for anti- $\alpha$ -enolase antibody
Normal control subjects	40	0 (0)
Behçet's disease patients	40	15 (37.5)
Patients with other rheumatic diseases		
Rheumatoid arthritis	6	1 (16.7)
Systemic lupus erythematosus	5	0 (0)
Wegener's granulomatosis	2	2 (100)

tients with SLE are associated with an active lesion of the kidney and vasculitis, and it has therefore been suggested that the AECA titer could be used as a measure of renal involvement (16).

Microvascular endothelial cells carry a specific antigen profile that is quite different from the profiles of the large vessels (17,18). In BD, vasculitis mainly involves capillaries and small vessels (2,3). Direct immunofluorescence of the lesions of BD patients mainly shows deposition of immunoglobulin on the walls of small veins (19). One study reported a higher prevalence of AECA when sera from BD patients were tested against microvascular endothelial cells as compared with human umbilical vein endothelial cells as the substrate (8). This finding provided the rationale for choosing HDMECs for the present experiments.

AECA-positive BD patients have a significantly higher frequency of active ocular lesions and acute thrombosis than do AECA-negative BD patients (7,8). We have also previously demonstrated that AECA-positive BD patients have a higher frequency of thrombophlebitis than do AECA-negative BD patients (20).

Histopathologically, BD has a characteristic vasculitis, presenting as an infiltration of CD4+ T lymphocytes around the vessels (21). Pretreatment of human endothelial cells with biologic response modifiers, such as interleukin-1 $\alpha$  (IL-1 $\alpha$ ) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), leads to an increase in the expression of intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1, and E-selectin molecules and, consequently, to an increase in the adhesion of T lymphocytes (14,22). In a previous study, we found that AECA-positive sera from BD patients led to changes in the expression of adhesion molecules on the cell surface of HDMECs and promoted the adherence of T lymphocytes to HDMECs and, thus, initiated or amplified inflammatory vascular injury (10). Our findings also suggested that IgM AECAs could play a pathogenetic role in BD by activating EC directly, and not via the production of TNF $\alpha$  or IL-1 $\alpha$  by HDMECs. In addition, as a signal transduction pathway, extracellular signal-regulated kinases 1 and 2 were involved in the expression of ICAM-1 on HDMECs stimulated by IgM AECA (23).

AECA has been detected at various frequencies in several diseases, but few studies have characterized the surface antigens against AECA. Moreover, there have been no other reports elucidating the antigens against AECA in BD. The human genome was completely sequenced recently, and as a result, many studies analyzing the function of various human genes are now



in progress. Proteomics is the most suitable method for these studies, because proteomics can analyze proteins by electrophoresis, and these results can be analyzed by several methods that identify the protein (24–26).

In this study, we produced an HDMEC protein map with ~2,000 protein spots on 2-D electrophoresis and an 18-cm IPG strip. We observed that many protein spots produced by BD patient sera, which on the 2-D immunoblot were between pH 5.0 and pH 8.0 (isoelectric point) and 44–50 kd, reacted with AECA-positive serum. Using the peptide fingerprint of the protein spot obtained by mass spectrometry, we were able to compare the protein with human  $\alpha$ -enolase and found that 7 of 17 peptides coincided within 30 ppm. Due to the high coincidence of 41% of the amino acid sequences, we were able to conclude that the protein reacting with AECA was a human  $\alpha$ -enolase of molecular weight 47 kd and of isoelectric point 7.0. Initially, we expected that the protein coinciding with AECA would be a previously unidentified protein. However, the protein was identified as  $\alpha$ -enolase, which has a known DNA sequence. Consequently, by making use of its known DNA sequence, it was not difficult to separate and refine the human  $\alpha$ -enolase using gene engineering.

The glycolytic enzyme  $\alpha$ -enolase is a homodimer of 47–48 kd. There is >90% homology among the  $\alpha$ -enolases of mammals (27). The  $\alpha$ -enolase enzyme catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate and is usually found in the cytoplasm. It exists as a multienzyme complex with other glycolytic enzymes, such as pyruvate kinase, phosphoglycerate mutase, muscle creatine kinase, and aldolase, in the cytoplasm (28,29). Although the mechanisms of  $\alpha$ -enolase expression in the cell membrane are not clear, enolase is expressed on the cell surface after exposure to an inflammatory stimulus. Enolase functions as the plasminogen receptor on the surface of various cells, such as epithelial cells, endothelial cells, and hematopoietic cells. The consequent binding of plasminogen to  $\alpha$ -enolase plays a crucial role in fibrinolysis. The role of this enzyme in systemic or invasive autoimmune disease was recently reported (30). Certain properties of  $\alpha$ -enolase, especially those related to surface expression and plasminogen binding, indicate that enolase may play an important role in the initiation of the disease process by modulating the pericellular and intravascular fibrinolytic system.

Antibodies against  $\alpha$ -enolase have been found in various inflammatory and immune disorders, such as cancer-associated retinopathy, ANCA-positive vasculitis, inflammatory bowel disease, discoid lupus erythem-

atosus, SLE, systemic sclerosis, endometriosis, primary membranous nephropathy, mixed connective tissue disease, and in autoimmune liver diseases (27,30,31). In SLE with nephritis, mixed cryoglobulinemia, and diffuse systemic sclerosis, antienolase antibodies react readily with renal and endothelial cell antigens, which express abundant  $\alpha$ -enolase and, thus, induce injury to those cells (27). The anti- $\alpha$ -enolase antibodies in the sera of patients with ANCA-positive vasculitis and inflammatory bowel disease react with antigens in the cytoplasm of neutrophils. In the present study, we found that antibodies against HDMEC  $\alpha$ -enolase were present in 37.5% of sera from 40 patients with BD. We also found that antibodies against HDMEC  $\alpha$ -enolase are present in patients with other systemic rheumatic diseases. Thus, the antibody response to HDMEC  $\alpha$ -enolase does not seem to be restricted to BD.

However, ELISA showed that the IgM antibody response to HDMEC  $\alpha$ -enolase was present only in the sera of BD and WG patients, and not in the sera of RA and SLE patients. Furthermore, all BD patients with anti- $\alpha$ -enolase antibodies were negative for RF, ANA, and ANCA, a finding that is inconsistent with the common antibody profiles observed in other rheumatic diseases. We might well conclude that this antibody response found in the sera of BD patients might be useful for the diagnosis of BD, since at present, there is no definitive diagnostic test for BD.

Three isoforms of enolase that display tissue-specific distributions have been described. Beta-enolase is found in skeletal and cardiac muscles,  $\gamma$ -enolase is expressed in neural tissue, and  $\alpha$ -enolase, the embryonic form, is ubiquitous, although it is most highly expressed in the kidney and thymus (32). Beta-enolase and  $\gamma$ -enolase share ~83% homology with  $\alpha$ -enolase. Despite the high degree of homology among the 3 enolase isoforms, the antibodies react only with the  $\alpha$  isomer, which suggests that these antibodies recognize a unique epitope on  $\alpha$ -enolase (33,34). In the present study, BD patient sera that were positive for anti- $\alpha$ -enolase antibody did not react with human  $\gamma$ -enolase. These results suggest that antibodies from BD patients react with a specific epitope structure of human  $\alpha$ -enolase.

Several previous reports have suggested that in autoimmune diseases, AECA is directed against class I and class II major histocompatibility complex (MHC) or ABO blood group antigens (35,36). However, antibodies against the MHC antigen in normal persons and in some autoimmune disease patients are often observed only during pregnancy or following transfusion. Yet, in the present study, many male patients were positive for this

antibody, and there was no history of transfusion or pregnancy among any of the antibody-positive BD patients. Previous studies have shown higher IgM positivity compared with IgG positivity for AECA in BD (20), and direct immunofluorescence has shown a predominance of IgM class among the immunoglobulins deposited in the venular walls of BD lesions (19,37). The present study also showed that the reactivity to HDMEC  $\alpha$ -enolase was detected only in IgM-positive BD sera. These results suggest an important role for IgM class AECAs in the pathogenesis of vasculitis in BD.

The presence of  $\alpha$ -enolase on the surface of streptococci may play a crucial role in the induction of autoimmune disease caused by streptococci (38). Serum samples from patients with acute rheumatic fever contain elevated levels of antibodies that react with both streptococcal and human  $\alpha$ -enolase. Also, Hsp48, one of the heat-shock proteins (HSPs), was identified as  $\alpha$ -enolase (39). Because HSPs are immunogenic molecules and can be expressed on cell membranes, their role in autoimmune and inflammatory diseases has been examined. A cellular immune response of T cells and a humoral response with the production of antibodies against HSP have been observed to occur in the course of those diseases (40). Autoantibodies may bind to the enolase on the surface of neutrophils and interfere with phagocytosis. Human enolase is expressed on the immune cell surface after an inflammatory stimulus, and it may react with anti-enolase antibodies. Generally, self-tolerance prevents an autoimmune response, but if this fails, the reaction of the enolase on the surface of immune cells with antibody may lead to opsonization or cell destruction, an increased inflammatory reaction, and tissue damage. Moreover, the high homology between streptococcal enolase and human enolase may facilitate the initiation and development of autoimmune reactions.

We cannot describe the precise role of  $\alpha$ -enolase in the pathogenesis of BD, nor can we explain the significance of the existence of antibody to  $\alpha$ -enolase in the sera of BD patients. However, we believe that this antigen can be used as a marker of BD. This is the first study to show that autoantibodies to the  $\alpha$ -enolase of HDMEC are present in the sera of BD patients. Possible explanations for the role of  $\alpha$ -enolase in BD include the histopathologic features of vasculitis, frequent retinal involvement, frequent reports of preceding bacterial infections (e.g., *Streptococcus*), the role of HSPs, and positive reactivity to ANCA. Thus, further studies on the relationship between  $\alpha$ -enolase and the pathogenesis of BD may yield important information about its pathogen-

esis. Moreover, it is hoped that our findings might aid in the development of new treatments for BD and be of value in studies of other autoimmune diseases in which vasculitis is a component. Further studies are necessary to elucidate the roles of this protein and circulating autoantibodies in BD, to determine the differences between BD and other autoimmune diseases in which vasculitis is a component, and to establish the role of *Streptococcus* in the pathogenesis of BD.

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