Identification of alpha-tropomyosin as a target self-antigen in Behçet's syndrome

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Behçet's syndrome is a multi-system inflammatory disease affecting mainly the oral and urogenital mucosa and the uveal tract. The etiology and pathogenesis of Behçet's syndrome are unknown, but autoimmune mechanisms are implicated. We initiated this work to identify self-antigens targeted by patients with Behçet's syndrome. We used patient sera to immuno-blot tissue lysates, and we found that some patients manifest antibodies to a 37-kDa band. The 37-kDa band was detected in extracts of skin, tongue, vagina, muscle and heart but not in brain, kidney, lung, liver, intestine and thymus. In-gel digestion and mass spectrometry revealed the band to be α-tropomyosin. Autoimmunity to α-tropomyosin can be pathogenic; immunized Lewis rats developed lesions in the uveal tract and skin, with features of Behçet's disease.

1 Introduction

Behçet's syndrome was described over 60 years ago [1]. The diagnosis is based on a typical clinical syndrome and the presence of a positive pathergy test [2]. The eyes, skin, joints, oral cavity, genital system, and blood vessels are usually involved; the central nervous system, heart, lung, kidney, and gastrointestinal tract are less frequently affected [1]. The disease is more prevalent in the Middle East and Japan. The pathogenesis of Behçet's is unknown. Numerous etiologies have been proposed, including environmental, infectious, and immunological factors; an autoimmune basis, characterized by circulating immune complexes and complement activation, has gained increasing acceptance [3]. Human lymphocyte antigen (HLA), Bw51, has been associated with Behçet's disease [4].

Previous work demonstrated the presence of antibodies to guinea-pig oral mucosal cells by immunofluorescence [5]. The nature of this cytoplasmic antigen was not defined. Recent work has demonstrated oligoclonal T cell expansion in patients with Behçet's syndrome, suggesting an antigen-driven immune response [6]. In uveitis patients with Behçet's syndrome, a proliferative T cell response was documented against retinal S-antigen and interphotoreceptor retinoid binding protein [7]. Some

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groups have found T cell proliferative responses to human heat shock protein (hsp) 60 and peptides derived from it [8], and others have found antibodies to *Yersinia*-derived hsp60 [9]. Lehner et al. [10] have characterized gamma-delta T cells reactive against several peptides of human hsp60 in patients with Behçet's syndrome.

We initiated this work to seek target antigens associated with the tissues involved that might be pathogenic in laboratory animals. We tested patient sera for the presence of antibodies to antigens found in lysates of various tissues. We identified a subset of patients with immune reactivity to a 37-kDa antigen present in the skin, tongue, vagina, muscle and heart rat tissue extracts. In-gel digestion and mass spectrometry revealed the antigen to be α-tropomyosin. The pathogenicity of reactivity to α-tropomyosin was demonstrated in a rat model. The contribution of these findings to the understanding of the pathogenesis of Behçet's syndrome is discussed.

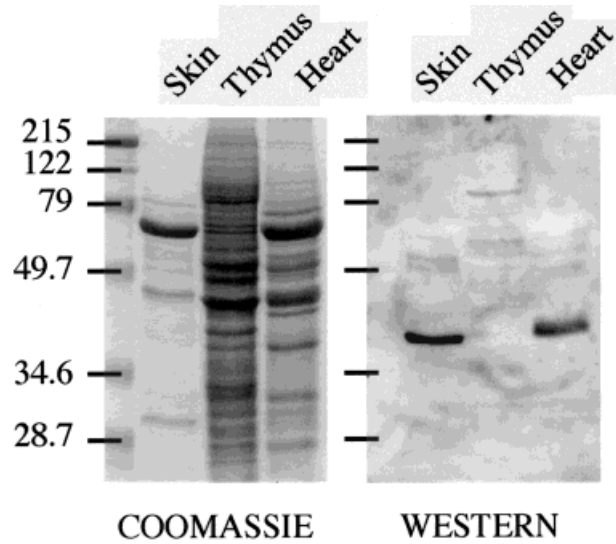## 2 Results

2.1 Behçet serum IgG reacts to a 37-kDa band

Lysates of rat tissues were run in polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose paper. To identify antibodies to tissue components, we analyzed the serum of a typical patient with active Behçet's disease. The serum was tested in immuno-blot against a panel of rat tissues and cell lines. Fig. 1 shows
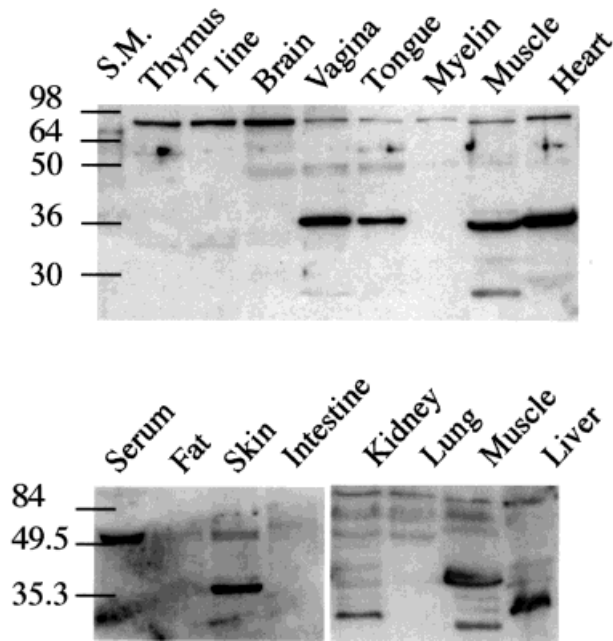
Fig. 1. Tissue expression of the 37-kDa band. Tissue lysates (100 µg protein) were separated by SDS-PAGE using 12% gels and electro-transferred to nitrocellulose membranes. Following incubation with the test Behçet's serum (1/1,000), the membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated anti-human IgG, and the antibody binding was measured using the ECL method. S.M indicates the positions of the pre-stained molecular size marker in kDa.

Fig. 2. Specificity of the serum reactivity. Tissue extracts were run in 12% gel. Half of the gel was processed for Western analysis (marked WESTERN), and the second half was stained with Coomassie brilliant blue (marked COOMASSIE). Details of the Western blot analysis are indicated in the legend to Fig. 1. The numbers indicate molecular weight size markers.

the pattern of reactivity of the serum to the different tissues. A 37-kDa band was seen in extracts of vagina, tongue, skin, muscle and heart but was absent from thymus, brain, myelin, fat, kidney, lung, serum and intestine. The 50-kDa band seen in the serum sample is probably

the immunoglobulin heavy chain detected by the secondary antibody. To examine the specificity of the serum binding, lysates of skin, thymus and heart were run in duplicate and half of the gel was stained with Coomassie while the other half was tested by immunoblot (Fig. 2). As can be seen, there was only a single major band of reactivity, and the thymus lysate that contained the largest number of bands was negative. To better characterize the distribution of the 37-kDa antigen, various human and rodent cell lines were tested. Of the lines tested, only clone 7 (a fibroblast clone of mouse origin) showed the band (Fig. 3).
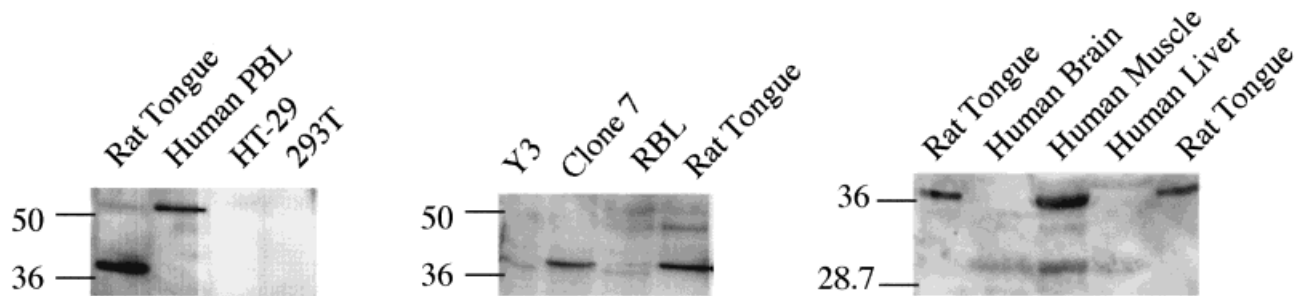
Fig. 3. Western blot analysis of the 37-kDa band in cell lines and human tissues. Cell lines were lysed and extracts were run in SDS-PAGE using 12% gels. Rat tongue extract was used as a positive control for the 37-kDa band. Human PBL denotes peripheral blood lymphocytes. HT-29 is a human colon carcinoma cell line; a human embryonic kidney cell line; Y3 is a rat myeloma; Clone 7 is a mouse fibroblast line; RBL is rat basophilic leukemia. In the right part of the figure, human brain, muscle or liver were tested. The 37-kDa band is seen in clone 7 cells and in human muscle.

Table 1. Patient characteristics

Patient	HLA B5	Clinical manifestations	37-kDa antibody
1	B5	OU[a)], GU, Skin	++
2	N.D.	OU, GU, Skin, Arthritis	
3	B-51	OU, GU, Skin, Arthralgia	
4	B-52	OU, GU, AU, Arthralgia	++
5	B5	OU, Skin,	
6	B-51	OU, GU, RV, AU, Skin	
7	B-51	OU, GU, DVT, Arthritis, EN, Pathergy	++++
8	Neg.	OU, GU, Skin, Arthritis, EN	
9	B-51	OU, GU, Skin, DVT, Arthritis	
10	B-51	OU, GU, Skin, SVT, Arthritis	
11	B-51	OU, GU, Pathergy Arthritis, CNS, EN	
12	B-51	OU, GU, Skin, AU, Arthritis	
13	B-51	OU, GU, DVT, SuVT, ArtT, Arthritis	
14	Neg.	OU, GU, Arthritis, EN	+++
15	B-51	OU, GU, Skin, AU, DVT, SuVT, Arthritis	

a) OU – oral ulcers, GU – genital ulcers, AU – anterior uveitis, RV – retinal vasculitis, DVT – deep vein thrombophlebitis, EN – erythema nodosum, SuVT – superficial vein thrombosis, ArtT – arterial thrombosis.

Fourteen additional sera from Behçet patients were tested in immunoblot against skin and tongue lysate (Table 1). Three additional Behçet's patients were found to be strongly positive for reactivity to the 37-kDa band. A similar analysis of 20 healthy blood donors and 16 patients with recurrent aphtous stomatitis was negative for antibodies to the 37-kDa band in Western blots (not shown). We could not correlate the presence of the 37-kDa band with any specific clinical manifestation of Behçet's syndrome.

2.2 The 37-kDa band is an autoantigen

We tested human tissues to verify that the antibody detected to the rat tissue antigen was an autoantibody. As can be seen in Fig. 3, the band was clearly present in human muscle lysate and was absent from human brain and liver. Thus, the pattern of tissue reactivity was similar

for rat and human tissues, indicating that the IgG antibody detected was an autoantibody.

2.3 The 37-kDa band is α-tropomyosin

To identify the 37-kDa antigen, we used the lysate of rat heart that showed the highest level of expression of the antigen. The lysate was subjected to anion exchange chromatography and was found to bind to quaternary ammonium (mono Q Sepharose fast flow Pharmacia Biotechnology, LKB, Sweden). The band was then eluted with 0.35 M NaCl (Fig. 4A), passed through a ConA column, and lyophilized. In the next step, the lyophilized material was dissolved in 100 μl of water and was separated by PAGE. The 37-kDa band was excised from the
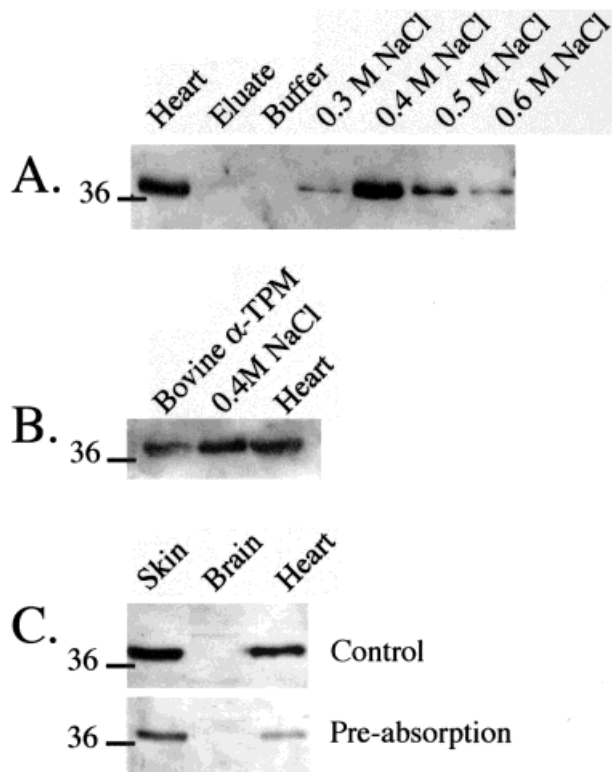
Fig. 4. (A) Anion exchange chromatography of the 37-kDa band. Heart lysate was separated using anion exchange chromatography. The 37-kDa band was eluted from the resin using 0.4 M NaCl. (B) In the middle part of the figure, commercial bovine α-tropomyosin (2 μg) was run in SDS-PAGE and immunoblotted with the Behçet's serum. The 37-kDa band was detected in the α-tropomyosin, similar to the heart lysate and the 0.4 M NaCl eluted material. (C) Reduced binding to the 37-kDa band after pre-incubation with bovine tropomyosin. In the lower part of the figure, binding of the serum to the 37-kDa band was tested in Western blots after pre-incubation of the serum with bovine tropomyosin.

Table 2. Rat α-tropomyosin peptides identified by mass spectrometry[a]

Peptide sequence	Peptide origin
LDKENALDR	Peptide 13–21
QLEDELVSLQK	Peptide 38–48
LKGTEDELDKYSEALK	Peptide 50–65
ATDAEDVASLNR	Peptide 78–90
IQLVEEELDR	Peptide 92–101
LVIIESDLER	Peptide 169–178
YSQKEDKYEEEIK	Peptide 214–226
SIDDLEDELYAQK	Peptide 252–164

a) The 37-kDa band was purified from heart lysate, and after in-gel digestion peptides were identified by mass spectrometry.

gel and was subjected to in-gel digestion and mass spectrometry. The peptide mass spectrometry revealed eight different peptides that together covered 30% of the rat α-tropomyosin sequence. This finding identified the protein to be rat α-tropomyosin (Table 2). To confirm that the serum reacted to intact α-tropomyosin, bovine muscle α-tropomyosin was obtained (Sigma). Two micrograms of the bovine protein were run in a gel in parallel to the heart lysate and the enriched fraction of the anion exchange chromatography. As can be seen in Fig. 4B, the Behçet's serum stained a band of identical size in both the tropomyosin and tissue preparations. In addition, we tested the ability of pre-incubation of the serum with bovine tropomyosin to inhibit the binding to the 37-kDa band. Fig. 4C shows that the pre-incubation resulted in a significant reduction of the intensity of the band from both skin and heart samples.

To test the reactivity of the serum against the human α-tropomyosin, we ran a gel containing rat skin, bovine tropomyosin, human muscle and 1 μg of recombinant skeletal muscle human α-tropomyosin. As shown in Fig. 5, the serum stained a band of identical size in all preparations. Thus, rat, bovine, and human α-tropomyosin are of the same electrophoretic mobility and were recognized by the Behçet's serum. From the intensity of the recombinant human tropomyosin band (containing only 1 μg), it is evident that the affinity of binding was highest to the human recombinant antigen. The cross-reactivity of the serum against a variety of α-tropomyosin sources is expected due to the highly conserved nature of the protein sequence: human and rat α-tropomyosin differ in only 2 of their 284 amino acids.
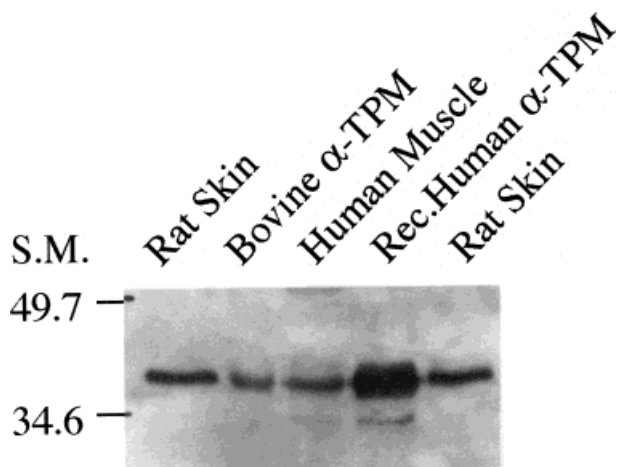

Fig. 5. Western blot of human recombinant α-tropomyosin. Extracts of rat skin, bovine α-tropomyosin (marked α-TPM), human muscle, and human recombinant α-tropomyosin (1 μg) were run in parallel and tested in immunoblot with Behçet's serum.

2.4 Pathogenicity of α-tropomyosin in the Lewis rat

To test whether induction of autoimmunity to α-tropomyosin might be pathogenic, we immunized Lewis rats with bovine α-tropomyosin in CFA. The immunized rats developed signs of anterior uveitis and an inflammatory skin infiltrate (Fig. 6, 7). Histological sections of the affected eyes showed marked thickening of the iris and uvea with mononuclear cell infiltration in and around the ciliary body (Fig. 6B, C). The skin showed a mixed cellular infiltrate with micro-abscess formation in the epidermis and peri-vascular and interstitial accumulation of inflammatory cells. Control rats injected with PBS/CFA emulsion did not develop uveitis (Fig. 6A) or skin inflammation (Fig. 7A). Histological analysis of tropomyosin/CFA-injected rats did not show inflammatory changes in the heart, skeletal muscle, stomach, kidney or ovary.

To test whether the observed changes could be due to activity of anti-tropomyosin T cells, a T cell line to the bovine antigen was prepared. Rats injected with the tropomyosin-specific line showed signs of anterior uveitis with thickening and infiltration of the ciliary body and iris, and posterior synechiae between the iris and the capsule of the lens (Fig. 6D).

Since the tropomyosin was purified from bovine tissue it was possible that the preparation contained other proteins in addition to tropomyosin. To verify that the pathogenic effects were due to specific tropomyosin autoimmunity, rats were immunized with synthetic tropomyosin
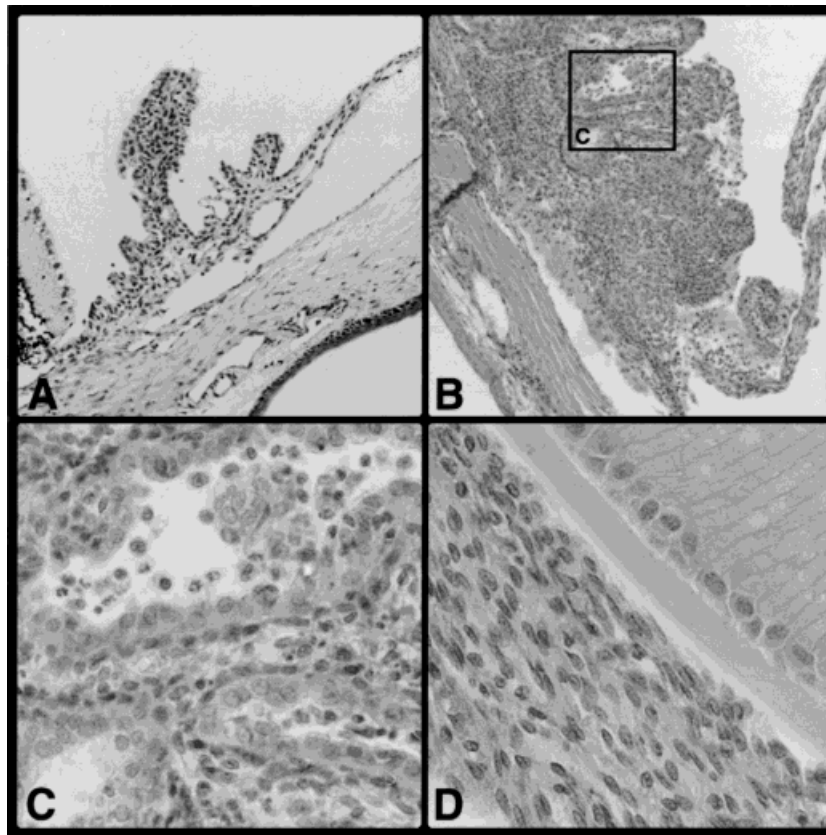
Fig. 6. Uveitis induced by immunization to α-tropomyosin. Lewis rats were immunized with tropomyosin in CFA and their eyes were examined 14 days later. (A) Ciliary body and iris from a rat injected with PBS/CFA the cornea is in the lower left part (Magnification X100). (B) Massively infiltrated ciliary body from a rat injected with tropomyosin in CFA (Magnification X100). (C) Magnification (X400) of the marked part on panel B, showing that the inflammatory infiltrate includes both mononuclear and polymorphonuclear leukocytes. (D) Infiltration and thickening of the iris 7 days after inoculation of tropomyosin-specific T cell line (X400).

peptides in CFA or were injected with peptide-specific T cell lines. As shown in Fig. 7, the rats immunized with peptide T2 in CFA showed signs skin inflammation similar to those seen in the tropomyosin-injected rats. The rats injected with T2 in CFA also showed a polymorphonuclear cellular infiltrate in the iris and ciliary body.

3 Discussion

Behçet's syndrome is a multisystem inflammatory disease of unknown etiology. The disease typically begins in the third or forth decade of life and affected patients suffer from recurrent oral and genital ulcers, uveitis and skin lesions [1]. The pathological characteristics include vasculitis of small vessels in the vicinity of the oral, genital, ocular and CNS lesions [11]. Neutrophils from patients with Behçet disease are overactive, with increased superoxide production, enhanced chemotaxis and

excessive production of lysosomal enzymes [8]. The pathergy reaction is composed of a dense mononuclear infiltrate in the skin appendages and around blood vessels composed mainly of CD4$^+$ T cells that stain for CD45RO and HLA-DR [12]. Possible pathogenetic triggers include viral or bacterial infection and environmental and autoimmune mechanisms [1].

Previous work on the nature of autoantigens recognized by patients with Behçet's syndrome have identified hsp60 reactivity [10], antibodies to retinal antigens in patients with Behçet uveitis [13] and anti-mucosal antibodies [5] that were not characterized. Here we report the presence of α-tropomyosin antibodies in some patients with Behçet's syndrome. The pattern of reactivity to this protein showed increased expression in tissues affected by the disease, suggesting a possible pathogenic link of the disease to these autoantibodies. Our studies in rats showed that immunization with
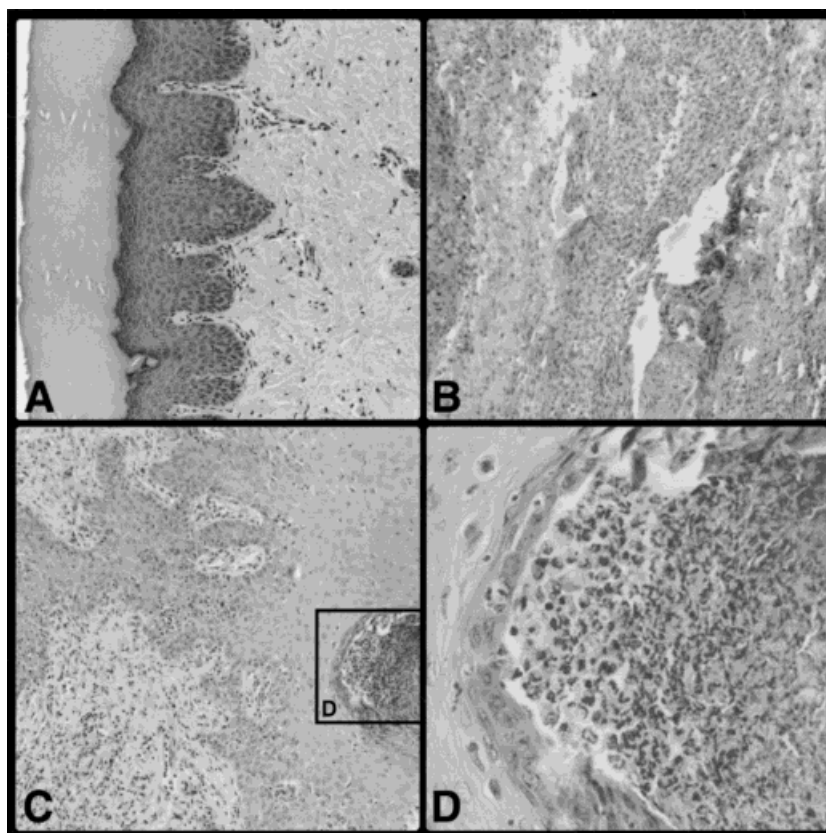
Fig. 7. Immunization to α-tropomyosin induces skin inflammation. (A) Skin from a PBS/CFA-injected Lewis rat (X100). (B) Subcutaneous tissue from the anterior paw of a rat injected with tropomyosin in CFA 14 days previously, showing a mixed inflammatory infiltrate. (C) Skin histology from a rat injected with peptide T2 in CFA 14 days previously, showing an inflammatory infiltrate in the dermis and an intra-epithelial micro abscess (X100), that is magnified in panel D (X400).

α-tropomyosin or some of its peptides may induce an autoimmune disease affecting the uveal tract and the skin. The rat skin pathology included a lymphocytic and neutrophilic infiltrate similar to that described in Behçet's patients [14], and the intra-epidermal pustules [12, 14] also seen in Behçet's skin inflammatory changes. Signs of anterior uveitis were seen in rats injected with tropomyosin or tropomyosin peptides in CFA and in the line inoculated animals.

The fact that only a subgroup of patients was positive for the antibody may indicate that the current clinical diagnosis may group together patients with different immuno-pathogenic mechanisms. In addition, as there is no definite diagnostic test, it is possible that some of the negative patients do not have Behçet's disease. Since recurrent oral ulcers are common and some of the skin and eye lesions are not specific for the disease, some patients may be falsely diagnosed as suffering from Behçet's disease.

The α-tropomyosin is a component of the cellular contractile apparatus [15] expressed in both muscle and non-muscle cells. The vertebrate α-tropomyosin gene consists of 15 exons; 5 exons are found in all transcripts, while 10 exons are alternatively used in different mRNA [16]. Mutations in the gene were found in some patients with familial hypertrophic cardiomyopathy [17]. The protein shows a high degree of homology in various species. Mice in which α-tropomyosin is knocked out manifest intra-uterine death at the age of 9.5–15.5 days [18].

The α-tropomyosin protein is a major antigen in allergic responses to invertebrates such as crustaceans (shrimp, lobster, crab, crawfish), arachnids (house dust mites), insects (cockroaches), and mollusks (squid) [19]. Autoimmune responses to α-tropomyosin have been previously reported in the sera of patients with ulcerative colitis [20], and in colonic intraepithelial lymphocytes [21]. However, other investigations did not detect this antibody reactivity in ulcerative colitis sera or in colonic lymphocytes [22, 23].

One of the leading theories about the pathogenesis of autoimmune diseases is the molecular mimicry hypothesis [24]. Thus, an immune response against a viral or bacterial antigen is proposed to activate a cross-reactive anti-self response that, in the absence of regulation, could result in autoimmune disease. In the case of Behçet's patients, an immune reaction to a pathogen or an allergic reaction to tropomyosin could activate an immune response to self-tropomyosin leading to the inflammatory manifestations of Behçet's syndrome. The results in rats showing that the induction of immune reactivity to tropomyosin results in uveal and skin inflammation indicates that this autoantigen could have pathogenic relevance in human disease.

The lack of inflammatory changes in the heart and skeletal muscle of tropomyosin/CFA-injected rats is intriguing in view of the high expression of the antigen in these organs. However, the biological rules that render an antigen pathogenic upon immunization to a certain tissue are not known. The study of autoantigens has traditionally progressed using a reductionist approach. After immunizing with tissue extracts to induce autoimmune disease (in the 60's to the 80's), the researchers chemically isolated whole antigens that could reproduce the disease: myelin basic protein (MBP) or proteolipid protein in EAE [25], thyroglobulin in thyroiditis [26], myosin heavy chain in myocarditis [27] and myositis, etc. Even in the best-studied autoimmune model EAE, it is not known why the inflammatory changes are limited to the CNS despite the fact that MBP is also expressed in the peripheral nervous system. Possible explanations for this phenomenon may be related to the potential of the auto-antigen to be presented by MHC class II molecules, which is related to the cellular compartment of protein degradation [28]. In addition, some tissues may be equipped with better protective mechanisms against autoimmune T cells, such as the expression of FAS ligand that induce apoptosis of potential auto-aggressive T cells [29].

The present study has several limitations. First, the auto-antibody to α-tropomyosin has been detected in only a minority of the examined patients. Potential reasons for the lack of reactivity were discussed above, and one has to consider the possibility that epitope spreading [30] may occur and shift immune reactivity to other antigens in tropomyosin-antibody negative Behçet patients [31]. Secondly, despite several attempts, we did not demonstrate T cell reactivity to α-tropomyosin in the peripheral blood lymphocytes of affected patients. Our experience in human autoimmune diseases such as multiple sclerosis, in which the autoantigens are known, highlights the difficulty of demonstrating T cell proliferation in primary cultures. Since the frequency of autoantigen-specific T cells in the peripheral blood is low [32], in the order of $1:10^5$–$1:10^6$ by limiting dilution analysis [32, 33], the signal-to-noise ratio in the human proliferation assay is quite low and the results obtained are not always reproducible. Thirdly, in order to further strengthen the causative role of α-tropomyosin autoimmunity in Behçet's pathogenesis, additional experiments are needed to show that the immune infiltrate in Behçet patients is enriched with antibodies or T cells reactive to α-tropomyosin.

Knowledge of the immune target in autoimmune disease is of potential therapeutic value as induction of tolerance to the autoantigen could lead to remission of autoimmune tissue damage. Tolerance can be induced by oral administration of auto-antigen [34] or parenteral injection [35] or by enhancing regulation by T cell vaccination [36]. In addition to the identification of a new target self-antigen in Behçet's syndrome, the methodology used in our study could be implemented in the analysis of other autoimmune diseases in which the focus of autoimmunity is not yet identified.

4 Materials and methods

4.1 Patient selection

Fifteen patients with Behçet's syndrome were randomly selected from the Rheumatology clinic of Rabin Medical Center, Beilinson Hospital. The clinical characteristics of the patients are shown in Table 1. Control sera were obtained from patients with recurrent aphtous stomatitis and from healthy blood donors. Sera were obtained after informed consent.

4.2 Animals

Inbred female Lewis rats were supplied from the Weizmann Institute animal breeding center and were used at 2–3 months of age.

4.3 Tissue isolation and lysate

Samples of tissues were homogenized using 1% NP40 in NaCl 0.9% Tris 50 mM EDTA 1 mM, containing protease inhibitors (0.4 mM PMSF, 10 mM benzamidine, 4 μg/ml pepstatin A, 4 μg/ml leupeptin, 4 μg/ml antipain, 4 μg/ml aprotinin). Brain, heart, lung, kidney, skin, intestine, peritoneal fat and liver tissue were homogenized with a tissue homogenizer in this buffer. Myelin was isolated from rat brain extract using a 0.8 M sucrose gradient. Brain extracts were loaded in ultracentrifuge tubes and covered with buffer containing 0.25 M sucrose. Centrifugation was performed at 35 Krpm using a Beckman SW 41 rotor, for 30 min at 10°C.

The myelin fraction from the interface 0.25–0.8 M was recovered and kept at –80°C.

4.4 Antigens

Bovine muscle α-tropomyosin was purchased from Sigma (Rehovot, Israel). Recombinant human muscle α-tropomyosin was a generous gift from Prof. Hitchkock-DeGregori [37].

4.5 Cell lines

The cell lines included anti-p277, a Lewis rat T cell line reactive against p-277, a peptide from the 337–360 sequence of the human hsp 60 kDa (sequence: VLGGGCAL-LRCPALDSLTPANED) [38]. Additional lines were Y3, a rat myeloma cell line; RBL, a rat basophilic leukemia cell line; HT-29, a human colon adenocarcinoma cell line; 293T, an embryonic human kidney clone; and clone 7, a mouse fibroblast clone.

4.6 Western blotting

Cell suspensions of thymocytes, cell lines, or tissue homogenates were lysed in lysis buffer. The protein concentration was determined using the Bio-Rad Dc protein assay, based on the Lowry method (Bio-Rad laboratories, Hercules, CA). From cell and tissue lysates, 100 μg of protein were loaded in each well. Following electrophoresis in 12% SDS gel in a mini-gel apparatus (Bio-Rad) the gels were electro-transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Gels were stained with Coomassie Brilliant Blue R-250 according to the manufacturer's protocol (Bio-Rad).

The nitrocellulose membranes were washed with distilled water for 5 min, and then blocked for 60 min using a blocking solution composed of 2% bovine serum albumin (Fraction V, Sigma, St. Louis, MO), 2.5% milk powder (Bio-Rad), Tris (Sigma) pH 7.5 10 mM, NaCl 150 mM and 0.02% thimerosal (Sigma). After three 10-min washes in PBS/Tween 20 (PBS/T; 0.02%, Sigma), patient sera (diluted 1/1,000) were incubated with the membranes in blocking solution for 60 min. Following another series of washes in PBS/T (three, 10 min), the membranes were incubated with a secondary antibody (peroxidase-conjugated goat anti-human IgG H+L, Jackson Immuno-Research, West Grove, PA) at a 1/2,500 dilution in blocking solution for 60 min. After another three 10-min washes, the membranes were incubated with the ECL reagent (for 2 min) and exposed to X-ray film for 15–90 s. For size determination, we used pre-stained broad-range protein standard markers (Bio-Rad). In the binding inhibition experiment, the serum (25 μl) was incubated during constant rotation with 40 μg of bovine tropomyosin in 200 μl of PBS; the control sample was incubated with PBS.

After overnight rotation at 4°C, both samples were used to stain tissue extracts run in the same gel and transferred to nitrocellulose.

4.7 Q Sepharose ion exchange chromatography

A heart tissue lysate was obtained from Lewis rats. The lysate was dialyzed overnight against 4,000 ml of 20 mM Tris-HCl pH 8 at 4°C, centrifuged at 9,000 rpm for 15 min, and the supernatant was collected. A free-flow column was packed to a volume calculated as a ratio of 10 mg protein/ 1 ml of Q Sepharose free-flow beads (Pharmacia LKB, Sweden). After equilibration of the column with three volumes of 20 mM Tris-HCl, the dialyzed lysate was loaded and the flow-through fraction collected. The proteins bound to the Q Sepharose column were eluted with increasing concentrations of NaCl (0.1–2.0 M). The different fractions were run on SDS-PAGE, and the 37-kDa band was examined in Western blots. The 37-kDa band was eluted with 0.4 M NaCl. In other experiments, we found the 37-kDa band did not bind to a Con A-Sepharose column (Pharmacia). The proteins that were eluted from the Q column and the Con A column (3.5 ml) were lyophilized and dissolved in 100 μl water and run in a 12% gel. The band at 37-kDa was cut and subjected to digestion and mass spectrometry.

4.8 In-gel digestion and mass spectrometry

Following gel destaining, the proteins were carboxymethylated in-gel using 100 mM iodoacetamide. The gel was then further destained in 50% acetonitrile (ACN) with 100 mM ammonium bicarbonate, cut to small pieces and dried. The gel was rehydrated with 10 mM ammonium bicarbonate pH 7.4 containing modified Trypsin (Promega, Madison, WI). After an overnight incubation at 37°C, the resulting peptides were eluted from the gel pieces with 60% acetonitrile with 0.1% TFA, and analyzed by LC-Mass Spectrometry. The peptides were resolved by HPLC on a 1X150 mm Vydac C-18 column, eluted with a linear gradient of 4–65% acetonitrile in 0.025 TFA, at 1%/min and a flow rate of 40 μl/min. The sample was microsprayed directly from the HPLC column into an electrospray ion trap mass spectrometer (LCQ, Finnigan, Thermo Instrument Systems Inc., Waltham, MA). The mass spectrometry analysis was done in the positive ion mode using repetitively a full MS scan followed by an MS/ MS experiment (collision-induced fragmentation) on the most dominant ion selected from the first MS scan. The MS and MS/MS data from the run were compared to the simulated proteolysis and fragmentation of the proteins in the "owl" database using the Sequest software (J. Eng & J. Yates, Univ. of Washington).

4.9 Rat immunization

Groups of five female Lewis rats were each immunized in both hind foot pads with 50 μg bovine α-tropomyosin

(Sigma) in complete Freund's adjuvant (CFA). CFA was composed of incomplete Freund's adjuvant (Difco, Detroit, MI) supplemented with 4 mg/ml of *Mycobacterium tuberculosis* H37Ra (Difco). The rats received 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA) intraperitoneally, on the day of immunization and 2 days after. Other groups of rats were injected with tropomyosin peptides (50 μg) in CFA, or T cell lines (15×10^6 i.p.). The rats were observed for signs of disease from day 10 post-immunization, or from day 4 after T cell line inoculation. At 14 days after immunization, the rats were sacrificed and their tissues were prepared for histological examination. The tissues were embedded in paraffin and sections were stained with hematoxylin and eosin.

4.10 Tropomyosin T cell line

Popliteal lymph node cells from rats immunized 14 days previously with tropomyosin in CFA were stimulated *in vitro* with bovine tropomyosin (10 μg/ml) in stimulation medium, as described [39]. To enhance the likelihood of immunogenicity [40], tropomyosin peptides were chosen from the rat sequence using an MHC motif. The peptide sequences were: T1: KKKMQMLKLDKENALDR (5–21); T2: LAEKKATDAEADVASLN (73–89); and T3: TNNLKSLEAQAEKYSQK (201–217). The T cell lines were expanded by stimulation every 10–12 days with antigen and irradiated thymocytes as antigen presenting cells [41]. Following four rounds of stimulation, the cells were analyzed for their specificity to the immunizing peptides in a proliferation assay, and for pathogenicity by i.p. inoculation (15×10^6) to naive Lewis rats.

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