

Patients with oral erosive and cutaneous lichen planus may have antibodies directed against the chronic ulcerative stomatitis protein antigen of 70-kDa

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ABSTRACT

Objective: The aim of this study is to verify whether stratified epithelium-specific antinuclear antibodies are present in the sera of patients with erosive oral lichen planus and cutaneous lichen planus.

Methods: We studied the pre-immune and immune serum of a rabbit immunized with a peptide corresponding to the N-terminus of the 70-kDa antigen chronic ulcerative stomatitis protein; sera from two patients, one with oral erosive lichen planus and one with cutaneous lichen planus who presented stratified epithelium-specific antinuclear antibodies at high titer; and a third serum from a patient with cutaneous lichen planus without stratified epithelium-specific antinuclear antibodies.

Results: We demonstrated that the protein bands recognized by the serum of the rabbit immunized with an epitope of chronic ulcerative stomatitis protein co-migrated by SDS-PAGE with the protein bands recognized by the serum of a patient affected by oral erosive lichen planus and by the serum of a patient with cutaneous lichen planus, both containing antibodies directed against a 70-kDa antigen.

Conclusions: Our results confirm that antibodies specifically directed against the chronic ulcerative stomatitis protein are not a distinctive marker of chronic ulcerative stomatitis, but may also be detected in oral erosive and cutaneous lichen planus.

KEY WORDS

chronic ulcerative stomatitis, erosive lichen planus, antinuclear antibodies

Introduction

Antinuclear antibodies (ANAs) distinctively directed against the nuclei of stratified epithelia (SES-ANA) characterize an entity described in 1990 called chronic ulcerative stomatitis (CUS), which clinically and histologically resembles oral erosive lichen planus (1–7). In addition, in patients with CUS, nuclear deposits of IgG in the epidermal cells are present on direct immunofluorescence.

In CUS, the SES-ANAs are directed against an antigen of 70-kDa (8, 9), the CUS protein (CUSP), which has been proved to be a member of the p53 family (10).

The close clinical and histological similarity of CUS and oral erosive lichen planus, however, suggested the possibility that SES-ANAs against the 70-kDa antigen may be also present in sera of patients with lichen pla-

nus, in which there are generally no ANAs. Thus, CUS could be a variant of lichen planus rather than represent a distinct entity.

In a previous study carried out in 2002 we found that 19 (13.8%) of 138 sera of patients with cutaneous, oral erosive, and non-erosive lichen planus had SES-ANAs in which, in nine cases (7/9 with cutaneous lichen planus), SES-ANAs were directed against a 70-kDa antigen band as it occurs in CUS sera. Only two of the nine patients had oral erosive lichen planus (11).

To assess whether SES-ANAs from the different forms of lichen planus – oral erosive and cutaneous – recognize the same protein (CUSP) as autoantibodies from patients with CUS, we performed the following study.

Material and methods

Rabbit immunization

An adult New Zealand rabbit was immunized according to the standard immunization protocol with a peptide of 14 amino acids (LENNAQTQFSEPPQYTNL) corresponding to the N-terminus of CUSP (10) and provided by Tib Molbiol (Advanced Biotechnology Centre, Genoa, Italy).

The peptide, conjugated with keyhole limpet hemocyanin and diluted in a bovine serum albumin adjuvant, was used to inoculate the rabbit once a month for 8 months. Each inoculation consisted of 500 µg of peptide. The rabbit was bled before the first inoculation, providing a pre-immune serum, and several times during the immunization protocol. In this study we used the 7th bleed (immune serum), which showed the highest anti-CUSP antibody titer.

Patients

Immunoblotting (IB) was used to compare the protein bands recognized by the serum of the immunized rabbit with the bands recognized by the sera from the following three patients.

Patient 1 was a 49-year-old woman with oral erosive lichen planus. Histopathology findings were consistent with lichen planus and DIF was negative. IIF using a stratified epithelial substrate (monkey esophagus) (ME) showed high titers of antibodies directed against the nuclear antigens of basal keratinocytes at a final titer of 1/320, whereas it was negative using HEp-2 2000 cells as a substrate. IB showed antibodies co-migrating at 70-kDa. This patient was therefore labeled a SES-ANA positive patient with oral erosive lichen planus.

Patient 2 was a 62-year-old man with cutaneous, but not mucosal, lichen planus (Fig. 1). The diagnosis of lichen planus was confirmed histopathologically and DIF showed deposits of IgG, IgM, and fibrinogen on cyto-

id bodies in the papillary dermis. IIF using ME showed ANA directed against the nuclear antigens of keratinocytes at a final titer of 1/320, whereas it was negative on HEp-2 2000 cells. IB showed that the antibodies co-migrated at 70-kDa. This patient was labeled an SES-ANA positive patient with cutaneous lichen planus.

Patient 3 was a 61-year-old woman with cutaneous lichen planus on her trunk and upper limbs. No mucosal lesions were present. Histology was consistent with lichen planus and DIF was negative. IIF did not disclose antibodies directed against nuclear antigens when both ME and HEp-2 2000 cells were used as substrates. IB was negative. This patient was called labeled an SES-ANA negative patient with cutaneous lichen planus.

Indirect immunofluorescence

IIF was performed on monkey esophagus (Alfabiotech, Rome, Italy) and on HEp-2-2000 cells (ImmunoConcepts, Sacramento, CA, USA) with sera diluted 1:20 in PBS. After a 25 min incubation, the slides were washed for 15 min with PBS and incubated with the secondary fluoresceinated antibody for 25 min. As secondary antibodies, fluorescein isothiocyanate-labeled goat anti-human IgG (Kallestad Diagnostic, Chaska, MN, USA) diluted 1:50 in PBS and anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:500 in PBS were used. After PBS washing, the slides were mounted in glycerin and observed through the fluorescence microscope.

Immunoblotting

Plates of keratinocytes at confluence were used to produce a total extract for IB. After washing with PBS, the keratinocytes were extracted with a buffer containing 65 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, and protease inhibitors (200 µM phenyl-methyl-sulfonyl fluoride, 1 µM leupeptin, 1 µM pepstatin, and 100 µM ethylenediaminetetraacetic acid). Keratinocytes were incubated for 30 min at 4 °C with the above buffer. The lysates were sonicated at 4 °C and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was stored at -20 °C and then used as the extract for IB.

The keratinocyte extracts were electrophoresed on 6% polyacrylamide minigel using the Laemmli method (12). Seven µg of extract were loaded on each well, separated by electrophoresis and blotted with Towbin buffer (TBS 10 mM Tris HCl, 140mM NaCl). IB was carried out overnight at 4 °C using rabbit and human sera diluted 1:100 in TBS/milk. Secondary antibodies were used for 30 min at room temperature. In detail, biotinylated goat anti-human IgG (Dako, Glostrup, Denmark) diluted 1:500 in TBS/milk was used for human sera; goat anti-rabbit IgG conjugated with alkaline phos-



Figure 1: Clinical picture of patient 2: cutaneous lichen planus: papules on the wrist.

phatase diluted 1:500 in TBS/milk was used for rabbit sera. A compound streptavidine-alkaline phosphatase (Dako, Glostrup, Denmark) diluted 1:3,000 in TBS/milk was added for 30 min at room temperature on nitrocellulose filters previously incubated with human serum. The alkaline phosphatase activity was revealed by BCIP/NBT (Boehringer, Mannheim, Germany). After each step, the nitrocellulose strips were washed with a buffer containing 10 mM Tris-HCl, 140 mM NaCl, and 0.1% Tween20 at pH 7.4.

Results

Our experiment yielded the following results. In IIF on ME, the rabbit immune serum revealed SES-ANA, whereas the pre-immune-serum did not. In IB, a protein band of 70-kDa recognized by the immune rabbit serum (Fig. 2, lane 1) was identical to the ones obtained from the SES-ANA positive oral erosive lichen planus serum (patient 1) and the SES-ANA-positive cutaneous lichen planus serum (patient 2) (Fig. 2, lanes 2, 3) by SDS-PAGE. The 70-kDa band was not found in the pre-immune rabbit serum or in the SES-ANA negative cutaneous lichen planus serum (patient 3) (Fig. 2, lanes 4, 5).

Discussion

In this study, the protein bands recognized by the serum of a rabbit immunized with an epitope of CUSP, as identified by Lee et al. (10), co-migrated by SDS-PAGE with the protein bands of the serum of a patient affected by oral erosive lichen planus and the serum of a patient with cutaneous lichen planus, both containing antibodies directed against a 70-kDa antigen. We con-

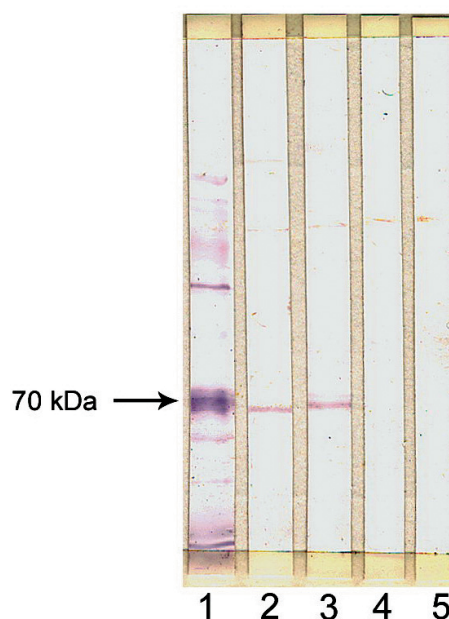


Figure 2: Immunoblotting on keratinocyte extract: the immunoblot showed a 70-kDa band with the immune rabbit serum (lane 1), the oral erosive SES-ANA positive lichen planus serum (lane 2), and the SES-ANA positive cutaneous lichen planus serum (lane 3). There was no reactivity at 70-kDa with the pre-immune rabbit serum (lane 4) or with the SES-ANA negative cutaneous lichen planus serum (lane 5).

clude, therefore, that the antibodies directed against a 70-kDa antigen found in patients with both the erosive and cutaneous forms of lichen planus are in fact directed against CUSP.

Our results confirm, therefore, that the antibodies specifically directed against CUSP are not a distinctive marker of CUS. Whether CUS could be distinguished from erosive lichen planus on the basis of other criteria than anti-CUSP antibodies and whether the presence of a 70-kDa band has clinical significance remains to be clarified. On the other hand, the clinical criteria distinguishing CUS from oral lichen planus (mainly erosive oral lichen planus) have still not been well described and only a few published pictures are available. As a result, the diagnosis of CUS is at present essentially immunologically-based when patients have unspecified clinical and histopathological oral ulcerative lichenoid lesions. Several circulating autoantibodies have been described in patients with oral lichen planus. They include those directed against CUSP and others: anti-desmoplakin I and II (13), anti-cardiolipin (14), anti-nuclear, anti-smooth muscle, anti-mitochondrial, anti-

thyroid anti-gastric parietal cell (15), and anti-basal cell cytoplasmatic (16). In most cases, however, the pathogenetic link between the oral lesions and the circulating autoantibodies is far from being demonstrated. In fact, both antigen-specific and non-specific mechanisms may be involved in the pathogenesis of oral lichen planus. Antigen-specific mechanisms include antigen presentation by basal keratinocytes and antigen-specific keratinocyte killing by CD8⁺ cytotoxic T-cells. Non-specific mechanisms include mast cell degranulation and matrix metalloproteinase activation. These mechanisms may combine to cause T-cell accumulation in the superficial lamina propria, basement membrane disruption, intra-epithelial T-cell migration, and keratinocyte apoptosis (17). The increased production of T cell cytokines and activated T cells, and the consequent apoptosis of keratinocytes, seem to be key events in the pathogenesis of this disease. On the contrary, autoantibodies, including SES-ANA, are unlikely to play an important role in the pathogenesis of lichen planus.

As stated in our previous article (11) and also demonstrated in this one, the only immunological differ-

ence between CUS and anti-CUSP-positive oral erosive and cutaneous lichen planus lies in DIF findings. In fact, in our two SES-ANA positive lichen planus patients, DIF did not reveal any IgG deposits in the epithelial cells. However, as Solomon et al. underline in their review (7), *in vivo* ANA can also be detected in other diseases; namely, idiopathic connective tissue diseases such as lupus erythematosus and scleroderma, or mixed connective tissue diseases. In these forms, however, ANA are present throughout the thickness of the epithelium and are not limited to the basal and parabasal layers as in CUS. This aspect could be the truly distinctive finding that characterizes CUS, rather than the antigen specificity. However, larger studies are needed to confirm our results.

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