Patients with Erosive Lichen Planus May Have Antibodies Directed to a Nuclear Antigen of Epithelial Cells: A Study on the Antigen Nature

Aurora Parodi, MD., and P.P. Cardo, Biol.D.

Department of Dermatology and Laboratory of Immunopathology, Research Center on Leprosy and Tropical Dermatology (C.I.R.LEP.), University of Genoa, Genoa, Italy.

Two patients with erosive lichen planus and latent HBV infection who had circulating antibodies directed to nuclei of epithelial cells are described.

The nature of such antigen has been investigated by indirect immunofluorescence, double immunodiffusion, counterimmunoelectrophoresis, enzyme-linked immunosorbent assay, enzyme treatments, and immunoblotting.

The antigen cannot be identified as RNP, histone, soluble nuclear protein, nDNA, or ssDNA. It may be a DNA protein complex and preliminary immunoblotting data support the thesis that it may be a multimolecular complex. *J Invest Dermatol 94:689–693, 1990*

Lichen planus (LP) is a relatively common skin disease of unknown etiology. Histopathology and immunohistochemistry suggest that cytotoxic T cells attack and destroy replicating keratinocytes that express some antigen on their membrane.

The nature of such antigen is far from being known, though it is possible that multiple agents, including viruses, are involved.

We describe two patients, affected by erosive LP of the oral mucosa, in whom epidermal nuclear staining in direct immunofluorescence (DIF) of involved skin and high-titer antibodies to the nuclei of epithelial cells suggested the presence of an antigen somehow linked to epithelial nuclei. We performed further studies aimed at assessing its nature and its possible etiopathogenetic role.

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Reprint requests to: Aurora Parodi, Department of Dermatology, University of Genoa, V. le Benedetto XV, 7, 16132 Genova, Italy.

Abbreviations:

BI: binding index
BSA: bovine serum albumin
CIE: counterimmunoelectrophoresis
DIF: direct immunofluorescence
EA: early antigen
EBV: Epstein-Barr virus
ELISA: enzyme-linked immunosorbent assay
ESR: eritrosedimentation rate
HBc: hepatitis B core
HBsAg: hepatitis B surface antigen
IIF: indirect immunofluorescence
LP: lichen planus
NHS: normal human serum
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
PMSF: phenyl methyl sulfonyl fluoride
RBC: red blood cells
SDS: sodium dodecyl sulfate
SNP: soluble nucleoprotein
TBS: Tris-buffered saline
VCA: viral capside antigen
WBC: white blood cells

MATERIALS AND METHODS

Case 1 A 64-year-old woman presented with a 12-year history of erosive lesions of the oral mucosa. At the age of 20 years she was diagnosed as having pulmonary tuberculosis and was treated for many years with streptomycin and PAS. At the age of 52 she developed arthralgia, pancytopenia, and splenomegaly. Chronic hepatitis with initial cirrhotic changes was diagnosed. At the age of 63, lilac papules developed on her legs and arms.

On examination, she disclosed erosive lesions of lower lip and cheeks and typical LP papules on her legs and arms. Laboratory tests revealed 3.96×10^6 RBC, 1880 WBC, 59,000 thrombocytes; hypergamma globulinemia 30.4% (normal values [nv] 11–20.5) with hyper-IgG (2750 mg/dl; nv 900–1000) and hyper IgA (605 mg/dl; nv 90–400); ESR 50 mm/h; prothrombin 68% (nv 80–120) and fibrinogen 185 mg/dl (nv 200–400). Total haemolytic complement activity was decreased (610 HU/ml; nv 700–1100) and rheumatoid test was positive (1:80). HBsAg was negative but antibodies anti-HBs and anti-HBc were present. Chronic hepatitis with cirrhotic changes was diagnosed with liver biopsy. Histologic examination of a papular lesion of the leg showed typical LP features with hypergranulosis, a bandlike lymphocytic infiltrate obscuring the dermoepidermal junction.

DIF of the papular lesions of the leg revealed deposits of IgG in a speckled pattern on epidermal nuclei (Fig 1) and granular deposits of IgM at the dermo-epidermal junction and IgM and C3 in the dermal vessel walls.

The serum of this patient was referred to as #135.

In indirect immunofluorescence (IIF), antinuclear antibodies were negative using rat liver as a substrate, but antinuclear IgG with a speckled pattern at the final titer of 1/5120 were present using monkey esophagus (Fig 2*A*,*B*).

Case 2 A 53-year-old woman was seen with a two-year history of erosive lesions of the mouth. On physical examination, the mucosa of her lower lip and cheeks exhibited large non-infiltrated erosions. On her left temporal area there was a 0.5-cm-wide nodule, clinically diagnosed as basal cell carcinoma.

Routine laboratory tests revealed: ESR 26 mm/h, 3800 WBC, hypergammaglobulinemia 24.6%. HBsAg was negative, but anti-HBs and anti-EBV IgG (VCA and EA) were present. Hepatic echogram was normal. A biopsy specimen of the lower lip showed LP

Figure 1. Direct immunofluorescence shows the speckle-patterned deposits of IgG within the epidermal nuclei (magnification ×235).

changes (Fig 3). The nodule of temporal area was excised and histopathology confirmed the clinical diagnosis of basal cell carcinoma.

The serum of this patient was referred to as #365.

DIF of involved skin (lower lip) revealed speckle-patterned deposits of IgG in epidermal nuclei and deposits of fibrinogen at the dermo-epidermal junction. The same pattern of particulate epidermal nuclear staining was shown in the basal cell carcinoma.

IIF did not demonstrate antinuclear antibodies using rat liver as substrate, but antinuclear IgG binding, especially the epithelial lower layers at the final titer of 1:10,240 and IgM at 1:320, were detected using monkey esophagus.

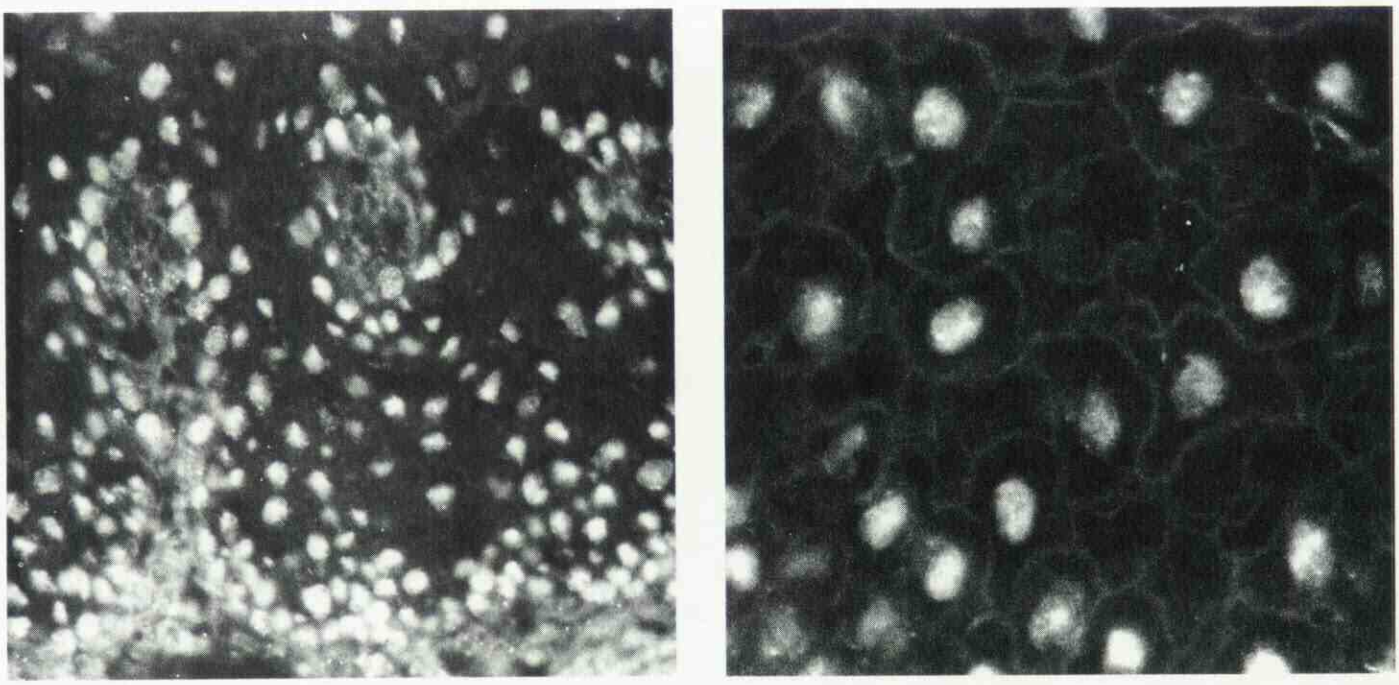
Further immunologic studies were endeavored to assess the nature of the nuclear antigen to which the antibody in the serum of both patients was directed.

IIF Additional IIF tests were performed by a standard technique using as substrates rat lip, normal human skin, calf esophagus, Hep 2 and BK from human squamous carcinoma cells, cultured mammary adenocarcinoma cells, cultured human keratinocytes, cultured rat kidney cells for detecting anti-mitochondrial antibodies, and *Crithidia luciliae* for anti-DNA antibodies.

Double Immunodiffusion Anti-Jo1, Scl-70, PM-1 antibodies were detected using the Ouchterlony double immunodiffusion method [1] and calf thymus extract as antigen.

Counterimmunoelectrophoresis (CIE) Anti-(U1)RNP, Sm, La/SSB, Ro/SSA, and Ku antibodies were determined by CIE using the method of Kurata and Tan [2] and Clark et al [3] and rabbit thymus and calf spleen extract [4] as antigens.

Preparation of Calf Esophagus Extract Calf esophagus was obtained from the local slaughterhouse immediately after the animals' death and kept on ice. All subsequent manipulations were carried out at 0–4°C. In a cold room, the esophagus epithelium was freed from surrounding tissues, washed, and weighted. Approximately 30 g of esophagus epithelium tissue were homogenized with 7 volumes of 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.2 (PBS), containing 0.25 M sucrose, 0.001% NaN_3, 0.012 M 2-mercaptoethanol, and 0.0002 M phenylmethylsulfonyl fluoride (PMSF, Sigma) freshly added. After repeated passages in a Waring blender set at high speed (with brief intervals for cooling) the pieces of unbroken tissue were discarded by filtration through four layers of gauze. The filtered homogenate was then spun at 1700 g_{av} for 30 min at 4°C. The pellet (P1) was resuspended with PBS using a Potter and kept at 4°C overnight. The low-speed surnatant was centrifuged for 1 h at 118,000 g_{av} and the pellet so obtained (P2) was treated as for P1. The clear high-speed surnatant was referred to as cytosol.



A B

Figure 2. *A:* Indirect immunofluorescence with calf esophagus as a substrate shows IgG binding nuclei of lower epidermal layers (magnification ×250) and *B:* under high magnification the pattern is speckled (magnification ×500).

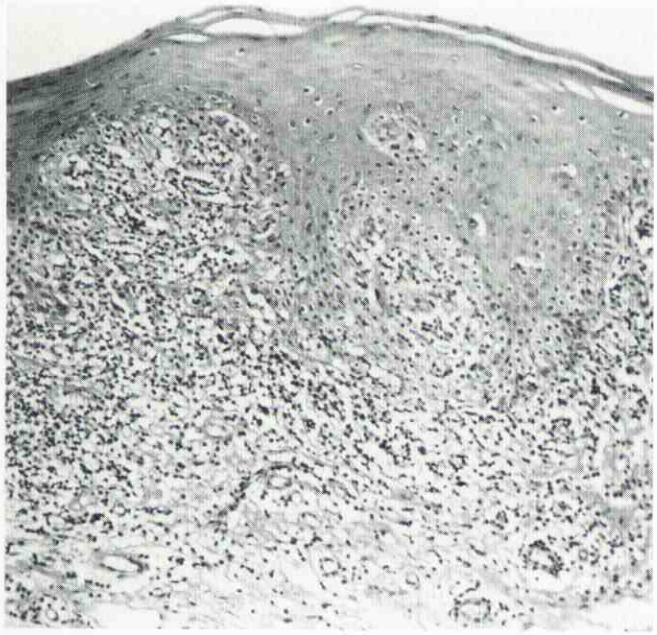


Figure 3. Histopathology of the lower lip shows lichenoid features.

Enzyme-Linked Immunosorbent Assay (ELISA) A solid-phase ELISA assay was developed using standard methodology [5]. Limbro 96-well microtiter plates (Flow) were coated overnight at 4°C with 100 μl of a fixed concentration of antigen preparation in 0.05 M carbonate buffer, pH 9.6. The unbound sites on the plate were blocked by the addition of 100 μL 0.5% bovine serum albumin (BSA, fraction V, Sigma) in carbonate buffer for 1 h at 37°C. The plates were then washed with 0.05% Tween 20 (Merck) in PBS using an automated apparatus (Titertek plate washer, Flow). Sera were diluted 1:100 in PBS containing 0.05% Tween 20 and 0.5% BSA, and incubated on the plate (50 μl/well) for 1 h at 37°C. The plates were washed again and then incubated with 50 μL/well of a 1:1000 dilution (in BSA-PBS-Tween) of conjugated antibody (horseradish peroxidase-labeled goat anti-human IgG or IgM, KpL) for 1 h at 37°C and washed again. One hundred μL of 2,2′-azino-di [3-ethyl-benzthiazoline sulfonate] (ABTS, KpL) mixed with H_2O_2 just before use was then added to each well and the chromophore development was measured at 414 nm with a Titertek Multiskan MCC reader (Flow). All measures were done in duplicate and repeated at least twice with complete reproducibility.

Enzymatic Treatments All digestions were carried out in PBS at 37°C for 1 h and stopped by chilling on ice and dilution in cold carbonate buffer. The resulting solutions were immediately used for coating the ELISA plates. Control samples were run in parallel using distilled water in place of the enzyme. For DNase and micrococcal nuclease digestion, bivalent cations (Mg^{++} and Ca^{++}, respectively) were added to a final concentration of 0.005 mole/l in the mixture. All of the enzymes (pancreatic deoxyribonuclease DNase I, pancreatic trypsin, yeast ribonuclease [RNase A], micrococcal nuclease, proteinase K) were obtained from Sigma.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting SDS-PAGE, using 10% acrylamide slab gel with 4% acrylamide stacking gel, was performed according to the method of Laemmli [6]. Samples were reduced for 3 min at 95°C with 0.0625 M Tris-Hcl buffer, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol and 10% glycerol. Samples were applied to the gel (1.5 × 83 × 67 mm) with 0.003% bromophenol blue as tracking dye and were run at 20 mA for 75 min. The following proteins were used as standards to calibrate the gel: bovine serum albumine (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), and beta-lactoglobulin (18,400). Polypeptides were electrophoretically transferred to nitrocellulose (0.45-μm pore size, Hoefer Scientific Instruments) for 3 h at 300 mA, according to the method of Towbin et al [7]. The nitrocellulose transferred proteins were either cut into strips and stained with amido black (0.1% amido black in 20% ethanol, 7% acetic acid) for detection of the marker proteins, or processed by immunostaining. Unstained strips were washed twice with 0.05% Tween 20 in 0.05 M Tris-HCl, pH 7.5, 0.2 M NaCl (TBST) on a rocking platform. They were then soaked in 5% BSA in TBST for 1 h at room temperature to saturate additional protein-binding sites. Antisera diluted 1:100 in 5% BSA-TBST reacted with the nitrocellulose by overnight rocking at 4°C. The strips were then washed twice for 10 min with TBST at room temperature. To detect bound antibodies, the strips were incubated with peroxidase-labeled anti-human IgG antibodies (KpL) diluted 1:200 in 5% BSA-TBST for 90 min at room temperature and washed twice with TBST and twice with Tris-buffered saline (TBS) without detergent for 10 min at room temperature. Immunoreactive bands were revealed by incubating the strips in 4-chloro-1-naphtol (Bethesda Research Laboratory) 0.5 mg/ml in TBS, containing 0.02% hydrogen peroxide.

RESULTS

IIF Antinuclear antibodies were not detected using BK, Hep 2, and mammalian carcinoma cells. Instead, they were present (IgG, 1:10,240; IgM, 1:320 for serum #365 and IgG, 1:5120; IgM, 1:40, for serum #135) when normal human skin, rat lip, calf esophagus, and cultured human keratinocytes were used (Fig 4). Under high magnification, they bind nuclei (Figs 3*B* and 4). Anti-mitochondrial and anti-nDNA antibodies were not detected.

Double Immunodiffusion and Counterimmunoelectrophoresis Anti-Jo-1, Scl-70, and PM-1 antibodies were not detected using double immunodiffusion. Anti-(U1)RNP, Sm, La/SSB, Ro/SSA, Ku antibodies were not detected using CIE.

Calf Esophagus Extract Differential centrifugation of the calf esophagus homogenate in isotonic sucrose yielded three fractions: a low-speed pellet (P1) containing nuclei and tissue debris, a high-speed pellet (P2) containing microsomial particles, and a clear surnatant (cytosol) containing all the non-particulate cellular constituents. P1 and P2 were then extracted in mild conditions using PBS, and P1s and P2s extracts were respectively obtained.

Initially, P1s, P2s, and cytosol fractions were examined by CIE, using the prototype sera (#135 and #365) and a pool of bench-sera (normal human serum, NHS) as controls.

Whereas NHS never reacted in CIE with the esophagus fractions, prototype sera gave precipitin spots in correspondence to the P1s wells. P2s and cytosol did not precipitate with sera.

The precipitation pattern of the prototype sera-P1s system was unusual, however, in that it was non-linear, with undefined borders and close to the serum wells, denoting a probable anionic polydisperse nature of the antigen. Identification was impossible in these conditions using the standard identity/non-identity approach with known monospecific sera.

Given the anionic behavior, the possibility that the prototype sera might contain anti-DNA antibodies was regarded as possible. Surprisingly, both sera proved negative with the *Crithidia luciliae* test, the Farr assay, and DNA-ELISA tests (data not shown).

On the other hand, a monospecific high-titer serum (MAC) reacting with either single- or double-stranded DNA gave precipitin lines when anodal wells were loaded with calf thymus DNA or P1s, strongly suggesting that P1s extract contained some kind of DNA. In addition, UV spectral analysis of P1s showed a high 260/280 absorbance ratio (>2.0) suggesting a nucleic acid-enriched character of this material (data not shown).

Enzyme-Linked Immunosorbent Assay Because of the peculiar CIE pattern and the limited amounts of antigenically active material in each preparation, we performed further experiments in the highly sensitive solid-phase assay ELISA. This was done by coating microtiter plates with the extracts and by incubating them

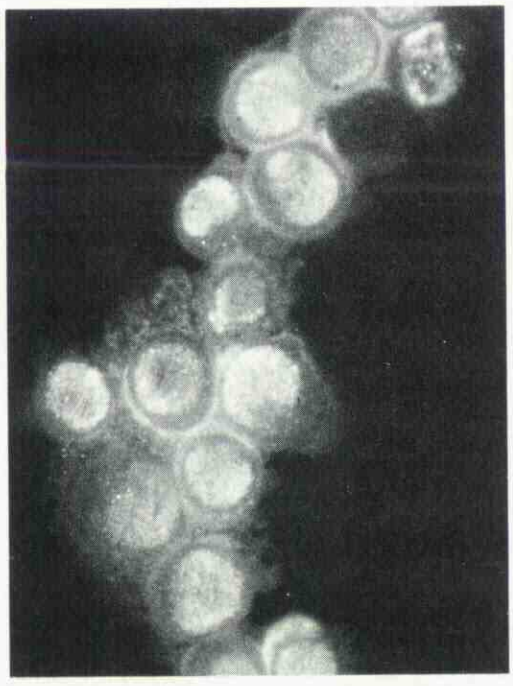


Figure 4. Indirect immunofluorescence with normal human cultured keratinocytes shows IgG binding nuclei with speckled pattern (magnification ×470).

with a fixed dilution (1 : 100) of prototype sera and with NHS used in our study.

For comparison purposes, approximately constant concentrations of extract, corresponding to a UV (280 nm) absorbance of 0.02–0.03, were used to coat the wells. The background was measured for each antigen preparation by omitting incubation with sera in some wells. As the background measures and those obtained in the wells incubated with NHS varied among preparations and comparing ELISA absorbances among plates was impractical, we arbitrarily scored the results as binding index (BI) defined as follows:

$$BI = \frac{\text{absorbance prototype serum} - \text{absorbance background wells}}{\text{absorbance NHS} - \text{absorbance background wells}}.$$

From a theoretical point of view, a nonspecific serum should give a BI close to 1, whereas, assuming a 0.5 standard deviation, a serum giving a BI > 2.0 should be specific for the coating substance with high probability ($p < 0.05$).

In fact, although the relative quantitative yields (ELISA absorbance/UV absorbance) varied among preparations, BI was reproducible among plates for a given preparation and among different homogenates.

Because both NHS and background may give high ELISA absorbance in the absence of serum, we performed parallel tests with sera preincubated with the same antigen preparation used to coat the plate. The proportion of BI not inhibited by preincubation was thought to represent nonspecific binding. As expected, inhibition never exceeded 30–40% with "crude" preparations, a result regarded as satisfactory in the absence of a better purification of this antigen.

When P1s, P2s, and cytosol fractions were compared by ELISA, the antigenic activity was found evenly distributed in all of the fractions, with a prevalence of P1s and cytosol (Table I). However, only in P1s fraction did preincubation of sera with a saturating concentration of antigen preparation inhibit binding more than 10%, suggesting a greater antigenic "purity" of this preparation. In this respect these data are in good agreement with CIE observations.

We concluded that binding observed in cytosol and P2s fractions was a nonspecific "noise" which was no longer investigated.

According to IIF observations, both #135 and #365 sera contained antibodies of IgG and IgM classes but IgG were the predominant isotype either from the BI's or from the inhibition's points of view. Serum #135 appeared to be more reactive than serum #365, but prior incubation with the antigen preparation caused serum #365 to inhibit BI more than #135.

For both #135 and #365 sera, BI and inhibition were at highest levels using anti-IgG conjugate, decreased using anti-IgM, and were minimal using polyvalent immunoglobulins.

Monospecific anti-Ro/SSA, anti-La/SSB, anti-Sm, and anti-(U1)RNP sera proved to react weakly with P1s extract at the 1 : 100 standard dilution (BI = 2.08, 1.15, 1.97, 0.99, respectively). On the contrary, prototype sera gave a signal clearly distinguishable from background even in very high dilutions (1 : 10,000), with a BI that grew exponentially by increasing serum dilution (data not shown).

Enzymatic Treatments P1s extract was treated with several enzymes before coating microtitration plates (Table II). Under the assay conditions, only DNase I (an endonuclease) and micrococcal nuclease (an exo- and endo-5′-phosphodiesterase) produced a marked reduction of BI for both sera. Trypsin slightly reduced BI with serum #365 and had a little increasing effect on BI with serum #135; in other experiments, proteinase K reduced BI (~17%) for serum #365. Interestingly, RNase A increased BI for both sera. Data in Table II were obtained with an anti-IgG conjugate, but qualitatively similar results were obtained using either anti-IgM or anti-human polyvalent immunoglobulins.

In all experiments, we failed in inhibiting BI obtained by the nuclease-digested antigen. The trypsin-digested antigen was inhibited for less than 10%, while the RNase-treated antigen was inhibited at the levels of control (data not shown).

Immunoblotting In order to investigate the antigenic components of the P1s extract, polypeptides were separated by SDS-PAGE, transferred to nitrocellulose paper, incubated with sera, and analyzed by immunostaining with peroxidase-labeled anti-human IgG antibodies. The results are shown in Fig 5.

A quite complex staining pattern is evident for both prototype sera: serum #365 was found to react with polypeptides of 105, 98, 74, 71, 39, and 29 Kd molecular weight (Fig 5, *lanes b* and *h*); serum #135 reacted with polypeptides of 98, 74, 71, 39, 32, 29, and 19 Kd (Fig 5, *lane c*)

Both sera recognize a doublet (two closely spaced bands) in the range of 70–75 Kd which was reproducibly present in different

Table I. Subcellular Distribution of the Antigenic Activity[a]

		P1s		P2s		Cytosol	
		BI	% Inh	BI	% Inh	BI	% Inh
#135	IgG	5.31	19.0	4.17	<10	5.69	<10
	IgM	4.22	12.7	4.15	<10	5.44	<10
#365	IgG	4.41	40.7	2.95	<10	4.59	<10
	IgM	3.19	33.8	2.70	<10	2.99	<10

[a] Values are given as BI and as percent of inhibition (see the text).

Table II. Enzymatic Treatment of the Antigenic Activity[a]

	#135	#365
Control	4.11	4.14
DNase I	2.75	2.42
RNase A	6.05	4.42
Trypsin	4.25	3.42
Micrococcal nuclease	2.21	1.47

[a] Values are given as BI (see the text).

extracts. The 70–75 doublet disappeared after trypsin treatment of the P1s extract, but was unaffected by DNase and RNase treatment (data not shown).

In addition, other faintly immunoblotted bands were shared by the prototype sera at 98, 39, and 29 Kd, while a 19-Kd band seemed to be recognized only by the serum #135 and a high-molecular-weight component, >100 Kd, was seen only with serum #365.

Our immunoblot findings for serum #135 and serum #365 are clearly distinct from staining patterns given by other relevant antinuclear specificities (Fig 5, *lanes d* through *g*) and NHS (Fig 5, *lane a*).

In addition, the immunoblot findings from a control patient who had erosive lichen planus, chronic postviral hepatitis, and negative IIF were different (data not shown).

DISCUSSION

Our findings may be summarized as follows:

1) Circulating antibodies in our patients with erosive LP are directed to an antigen that is present in epithelial cells of several mammal species.

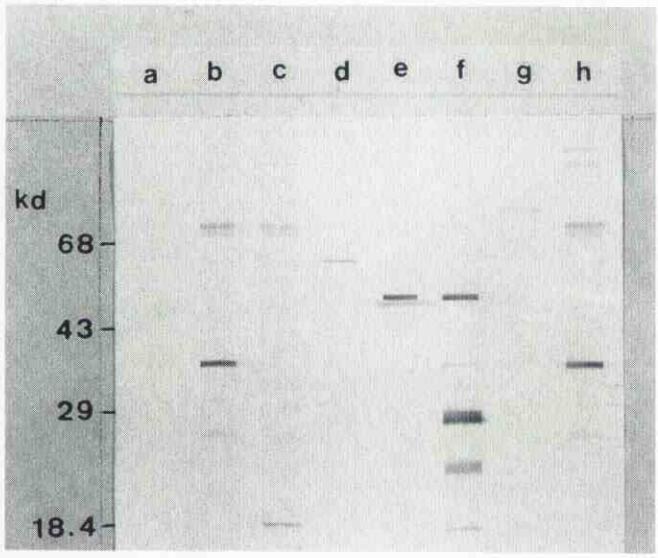


Figure 5. Immunoblots of calf esophagus P1s extract separated on 10% SDS-PAGE. The sample was loaded onto the slab gel in a single well that extended across the gel width. After electroblotting the nitrocellulose sheet was probed simultaneously with several sera, by a multiple-incubation chambers device (Mini Deca-Probe, Hoefer Sci. Instr.). *Lane a,* NHS; *lane b,* serum #365; *lane c,* serum #135; *lanes d* through *f,* CIE-monospecific sera anti-Ro/SSA *(d),* anti-La/SSB *(e),* anti-Sm/(U1)RNP *(f); lane g,* serum MAC anti-DNA; *lane h,* serum from the patient referred to as case 2 (serum #365), collected three years later. Molecular weight markers derived from a parallel electrophoresis are shown on the left.

2) Such an antigen cannot be identified as RNP as the speckled pattern in IIF and in vivo IgG binding observed in epidermis could have suggested. In fact, CIE findings were negative and substrates other than epithelial gave negative IIF results.

3) On the other hand, it can be recognized neither as a histone nor as a soluble nucleoprotein according to Tan et al [8] because (i) its pattern in IIF was speckled and not homogeneous as SNP and histone show; ii) SNP can be found also in the liver cells and this was not the case in our patients; iii) the conditions and the tissues in which SNP can be extracted are different from those we have used.

4) The antigen activity appears to be mainly affected by enzymes that break DNA, and secondarily by enzymes that hydrolyze proteins, while RNA seems to be an obstacle to the accessibility to the epitope.

In addition, the antigen in our patients cannot be an ordinary DNA molecule for we failed in finding it in other tissues, such as rat liver and calf thymus.

Yet, anti-nDNA and anti-ssDNA antibodies proved to be able to bind it.

5) By immunoblotting, the prototype sera proved not to be identical as they reacted to many polypeptides, but they shared the reaction to the same doublet in the range of 70–75 Kd and one of them kept doing so for three years. The clinical relevance of the other bands cannot be established at present, though it may depend on individual characteristics unrelated to lichen planus.

It may be, therefore, that we are dealing with a DNA-protein (non-histone) complex for which immunoblotting data support the thesis that they are multimolecular.

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