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Autoantibodies to human nuclear antigen(s)—HNA—in connective tissue diseases and other disorders

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SUMMARY

Autoantibodies reacting with nuclear antigen(s) on human cells (HNA) with weak or without reactivity on nuclei of other species have been found by the indirect immunofluorescence technique used in routine tests for the diagnosis of autoimmune diseases. Precipitin lines were obtained by counterimmunoelectrophoresis (CIE) only when human lymphocyte extracts were used and not with rabbit thymus acetone powder. By comparison with reference sera, the autoantibodies directed to HNA were found to be different from SSA/Ro antibodies and did not give the fluorescence pattern of anti nuclear mitotic apparatus (NuMA) antibodies on HEp-2 cells. The prevalence of sera with anti-HNA antibodies not associated with other antinuclear antibodies (ANA) is low (about 0·7% of ANA found in routine assay). In association with ANA of other specificities, the prevalence of anti-HNA antibodies, demonstrated after absorption of sera with rat liver acetone powder, was higher (about 1% of ANA positive sera). By treatment with physicochemical agents and enzymes, the HNA was found to be a DNA (glyco)-protein complex extractable with saline solution, resistant to 56°C for 6 h and stable at pH values ranging from 3 to 10. Anti-HNA antibodies were found in patients with mild connective tissue diseases, but also in idiopathic interstitial pneumonia and in chronic hepatitis.

Keywords autoimmunity antinuclear antibodies human nuclear antigen(s)

INTRODUCTION

Antinuclear antibodies (ANA) are generally known to be neither organ-nor species-specific. In the last few years, however, restricted specificities have been reported and confirmed by many Authors. Auto-antibodies reacting only with granulocyte-specific nuclear antigens have been described as characteristic of rheumatoid arthritis (Faber & Elling, 1966; Elling, Graudal & Faber, 1967; Faber, 1976), but have also been demonstrated in various connective tissue diseases or other disorders and even in healthy subjects (Faber & Elling, 1966; Smalley, Mackay & Whittingham, 1968; Ullman *et al.*, 1974; Vasey & Kinsella, 1977; Wiik, 1976). These organ-specific antibodies have no species restriction.

More recently it has been demonstrated that some antinuclear antibodies are directed against nuclear antigens from human cells, scarcely represented in the nuclei from other species. To our knowledge such a peculiar specificity has only been demonstrated for anti-SSA/Ro antibodies and for antibodies reacting with the nuclear mitotic apparatus (NuMA protein). SSA/Ro is an antigenic

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material of nuclear origin, but is also present in the cytoplasmic fraction of human cells (Clark, Reichlin & Tomasi, 1969; Alspaugh & Tan, 1975) and antibodies directed against such material are usually revealed by counterimmunoelectrophoresis using spleen cell cytoplasmic fractions. Anti-NuMA antibodies are detected by indirect immunofluorescence (IF) technique on human epithelial cell lines 2 (HEp-2) and on HeLa cell line, but not on proliferating cell lines from other species (McCarty *et al.*, 1981; Price, McCarty & Pettijohn, 1984). Studies on these peculiar antibodies are of value not only in order to identify nuclear antigens, their function and distribution in nuclei but also to discover clinical and/or serological subsets of connective tissue diseases.

We have recently had the opportunity of identifying another antibody reacting only or chiefly with human nuclei. The main characteristics of these antibodies and related antigen(s), tentatively defined by us as 'human nuclear antigen(s)-HNA' and their relationship to the patients' diseases are reported in the present study.

MATERIALS AND METHODS

Sera. About 600 blood samples are examined monthly by indirect IF technique for the screening of organ-and non organ-specific autoantibodies at the Allergology and Clinical Immunology Service of Florence University. Sera are obtained within three hours after collecting blood samples and stored at 4°C while the routine tests are performed. Aliquots are stored at −20°C for further analysis.

Immunofluorescence studies. Routine IF tests are performed on 5 μm cryostatic sections of human thyroid and gastric mucosa and of rat liver and kidney according to Roitt & Doniach (1969) using fluoresceinated antisera to human gammaglobulin from sheep (Wellcome Diagnostics, Dartford, UK). For special analysis fluoresceinated monospecific antisera to human IgG, IgA, IgM, C3, C4 (Wellcome Diagnostics, Dartford, UK) are used. The samples are examined by epifluorescence optics on a Leitz Orthoplan Microscope.

For the studies on antibodies directed against human nuclear antigens the following cryostatic sections were also used: kidney, liver, adrenal gland, parotid gland, skin, striated muscle, amniotic membrane of human origin from surgery or biopsy and kidney and liver from rabbit, guinea pig, rat, mouse, pig, cow. IF tests were also performed on human peripheral lymphocytes and on HEp-2 cells. Anti-dsDNA antibodies were detected by the Crithidia Luciliae method.

Absorption assays. Twenty-three sera with ANA reacting chiefly with HNA were absorbed with acetone powder from rabbit thymus, rat liver, horse lung and with human tonsil lymphocyte extracts. Absorption tests were also performed on 640 ANA positive sera with rat liver acetone powder.

The samples, diluted 1:10 in saline, were absorbed with appropriated amounts of the above mentioned materials at 37°C for 1 h and overnight at 4°C under continue mixing and then examined by IF on rat liver and human thyroid cryostatic sections.

Counterimmunoelectrophoresis (CIE). Was carried out according to Bunn, Gharavi & Hughes (1982). Rabbit thymus extract (RTE) (Pel-Freez, Rogers, Arkansas, USA) or human lymphocyte extract (HLE) were used as antigens. Human lymphocytes obtained from tonsils were purified on Ficoll-Hypaque gradient according to the method of Boyum (1968). After washing, the lymphocyte suspension was adjusted to $1{\cdot}4 \times 10^9$ cells/ml in phosphate buffered saline (PBS) pH 7·4 and sonicated. The soluble material was extracted by gentle mixing overnight at 4°C. The mixture was then centrifuged (18,000 ***g*** for 50 min at 4°C), the protein concentration measured by the Lowry method, adjusted to 10 mg/ml and stored in aliquots at −80°C until use (within a few weeks).

RTE was obtained according to the method described by Kurata & Tan (1976) and adjusted to 20 mg/ml in PBS.

The reference sera for anti Sm, RNP, SSB/La, SSA/Ro, SL and Jo-1 were kindly donated by Mr C.C. Bunn (Rheumatology Unit, Hammersmith Hospital, London, UK).

Physicochemical treatments. HLE was exposed at 37°C or 56°C for 30 min or 6 h and at various pH values. For the latter purpose HLE was dialysed against buffered solutions with pH values ranging from 4 to 11 for 18 h at 4°C and then against PBS pH 7·4 with several changes.

Digestion of HLE with enzymes (DNase, RNase, trypsin, pronase and neuraminidase) was performed for 30 min at 37°C. The reaction with trypsin was blocked by rapid cooling.

Human thyroid cryostatic sections were exposed at 37°C or 56°C for 30 min and 6 h before testing with IF techniques. The chemical treatment was performed by layering the following solutions on the sections fixed in ethanol: buffered solutions at pH values ranging from 2 to 11; 0·1 M sodium periodate ($NaIO_4$) in phosphate buffer (PB); 2 M sodium chloride; PBS; PB. After exposure for 30 min the slides were washed and used for IF tests. The sections were also treated with nucleolytic enzymes (15 min at 37°C) and with the other enzymes (10 min at room temperature). The enzyme solutions, in PB Ca^{2+} Mg^{2+} (0·006 M), were used at the following dilutions: DNase and RNase 100–200 μg/ml; trypsin and pronase 0·5–20 μg/ml; neuraminidase 0·01–1 U/ml.

RESULTS

By the IF technique, autoantibodies reacting only with nuclear antigen(s) of human cells, with weak or without reactivity on rat kidney and liver nuclei used in routine screening, were found in 23 out of 3,450 ANA positive sera (0·66%) of about 23,000 sera examined during the past 3 years.

As reported in Table 1, most patients were affected by mild connective tissue disorders. The ANA titre, evaluated by doubling dilutions on human thyroid cryostatic sections, ranged from 1:20 to 1:1.280.

All sera gave a bright fluorescence with reticular-peripheral pattern on nuclei of human thyroid and gastric mucosa (Fig. 1). The nuclear fluorescence pattern was peculiar and easily distinguishable from other patterns previously described. Nuclear fluorescence was obtained on all the human tissues examined, on human peripheral lymphocytes and HEp-2 cells.

Weak positive reactions on nuclei from other species, not exceeding the titre of 1:80, were observed only with 7 of 23 sera. The positive reactions on nuclei of other species were always abolished by absorption with rabbit thymus acetone powder, while the reactivity with human nuclei was never suppressed by such treatment even in the presence of low titre antinuclear antibodies. Similar results were also obtained by the absorption of sera with acetone powder from rat liver and horse lung. The treatment of sera with extracts of human tonsil lymphocytes induced the disappearance or at least a strong reduction in the titre of nuclear fluorescence on human cells.

Anti-dsDNA antibodies were never found. Anti-smooth muscle, anti-mitochondrial and anti-thyroid microsomal fraction autoantibodies were found, separately, in three out of the 23 sera examined and anti-immunoglobulin activity was found in eight out of 18 sera tested.

Anti-HNA autoantibodies were always of IgG class; eight and two sera also contained IgM and IgA anti-HNA antibodies respectively. *In vitro* complement fixing capacity was demonstrated in four out of the 14 sera tested.

Sera from 15 patients were examined twice or more at different intervals ranging from 3 months to 2 years and the immunofluorescence pattern was never found to be changed.

We were unable to obtain positive results by CIE against RTE. Sixteen sera gave a sharp precipitin line when HLE was used as antigen. They were shown to be identical to each other and different from all the reference sera used, as demonstrated by pairing assays (Fig. 2). Seven sera, with ANA titres ranging from 1:20 to 1:620 on human thyroid sections, did not show precipitin lines.

The results obtained by physicochemical and enzymatic treatment of HLE and thyroid cryostatic sections are shown in Table 2 and 3. By these experiments we have demonstrated that the HNA is resistant at 56°C for 30 min and weakly sensitive at 56°C for 6 h. It is soluble in saline but not in solutions at low ionic strength and stable at pH values ranging from 3 to 10. It is resistant to ethanol and 2 M NaCl and weakly sensitive to 0·1 M $Na\ IO_4$. Positive immunofluorescence reactions and precipitin lines against HLE were abolished by treatment with DNase, pronase, trypsin but were unaffected by RNase and weakly reduced by neuraminidase.

Fourteen out of 640 sera positive for ANA, examined after absorption with rat liver acetone powder, showed positive reactions only for human nuclear antigens. Six gave precipitin lines by CIE only against HLE, while five gave positive reactions also with RTE; three sera did not give precipitin

Table 1. Serological and clinical details of 23 patients in whose sera antibodies to HNA were found

Case no.	Sex	Age	Clinical diagnosis	ANA titre on: HT	ANA titre on: RL	Other autoantibodies	CIE against HLE	Remarks
1	F	30	Cutaneous Lupus	80	Neg.	—	anti-HNA	—
2	F	41	Cutaneous Lupus	320	40	—	anti-HNA	Alopecia
3	F	45	Cutaneous Lupus	640	40	—	anti-HNA	Raynaud's phenomenon
4	F	48	Cutaneous Lupus	320	Neg.	—	Neg.	Raynaud's phenomenon
5	F	56	Sicca Syndrome	320	Neg.	IgM RF	anti-HNA	Thrombocytopenia
6	F	54	Sicca Syndrome	160	Neg.	IgM RF	anti-HNA	Lymphoma
7	M	48	Sicca Syndrome	80	Neg.	IgM RF	Neg.	—
8	M	53	Rheumatoid Arthritis	320	Neg.	IgM RF	anti-HNA	—
9	F	28	Rheumatoid Arthritis	20	Neg.	IgM RF	Neg.	—
10	F	43	Rheumatoid Arthritis	640	10	IgM RF	anti-HNA	Diabetes mellitus
11	F	45	UCTD	640	Neg.	—	anti-HNA	Relapsing haematuria
12	M	50	UCTD	40	Neg.	—	anti-HNA anti-SSA/Ro	Hypertension
13	F	20	UCTD	40	Neg.	anti thyroid microsomes	Neg.	Chronic urticaria
14	F	28	UCTD	80	Neg.	—	Neg.	—
15	F	75	CAH	160	10	anti-SMA	anti-HNA	Chronic urticaria
16	F	34	PBC	160	Neg.	anti-SMA, AMA	anti-HNA	Diabetes mellitus
17	F	57	Idiop. Interst. Pneumonia	640	40	—	anti-HNA	—
18	F	51	Idiop. Interst. Pneumonia	160	Neg.	—	Neg.	—
19	F	78	Idiop. Interst. Pneumonia	20	Neg.	—	Neg.	—
20	F	56	Polymyositis	640	10	—	anti-HNA	Achrosclerosis
21	F	44	Dermatomyositis	320	Neg.	—	anti-HNA	—
22	F	35	A.T.P.	40	Neg.	IgM RF	anti-HNA anti-SSA/Ro	Photosensitivity
23	F	31	SLE	1280	80	Undefined anti 'ENA'	anti-HNA	—

HT Reciprocal of the titre of antinuclear antibodies (ANA) on human thyroid cryostatic sections; RL Reciprocal of the titre of ANA on rat liver cryostatic sections; HLE human lymphocyte extract; CAH Chronic active hepatitis; PBC Primary biliary chirrosis; UCTD Undefined connective tissue disease; A.T.P. Autoimmune thrombocytopenic purpura; IgM RF Rheumatoid IgM factor determined by the Latex test (Hyland); SMA Smooth muscle autoantibodies; AMA Anti mitochondrial autoantibodies.

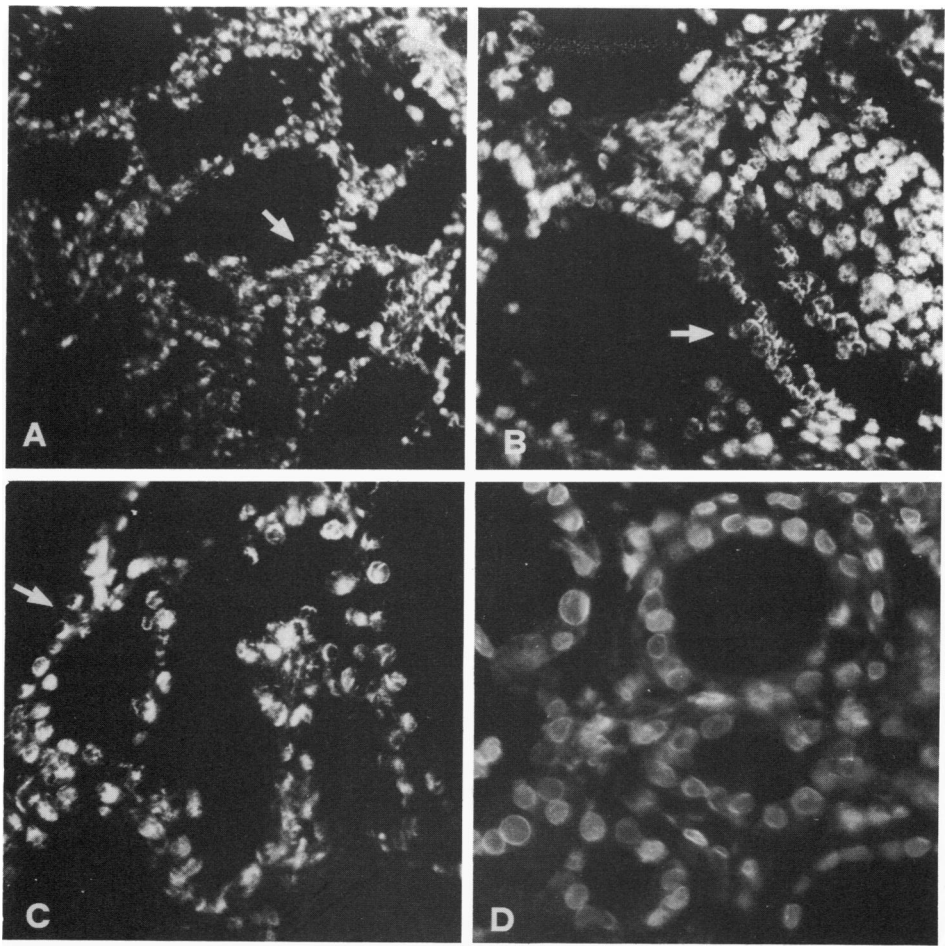

Fig. 1 Immunofluorescence pattern of anti-human nuclear antibodies (HNA) on human thyroid cryostatic sections. Note the peripheral and/or reticular aspect of fluorescence, particularly evident in the nuclei indicated by arrows. (A: × 240; B & C: × 384). The 'classic' peripheral pattern on human thyroid is shown in D (× 384).

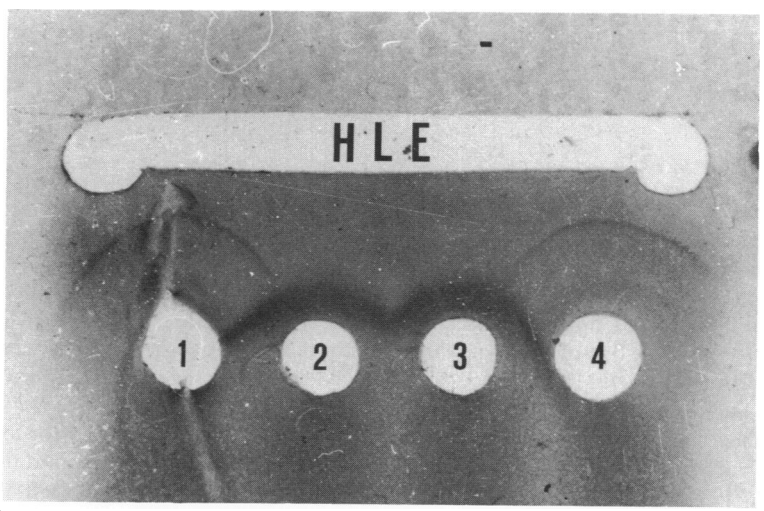

Fig. 2 Reaction of an extract of human lymphocytes (HLE) with sera specific for Human Nuclear Antigen(s) (2 & 3) and for the Ro antigen (1 & 4). 2 and 3 have a reaction of identity and their line of precipitation gives a reaction of non-identity with the precipitation lines of 1 and 4.

Table 2. Effect of physicochemical treatment on HNA.

Technique	37°C 30 min	37°C 6 h	56°C 30 min	56°C 6 h	PB 30 min	PB 18 h	PBS 30 min	PBS 18 h	pH stability	Ethanol	NaCl (2 M)	Na IO_4 (0·1 M)
IF	R	R	R	r	I	I	I	S	3–10	R	R	wr
CIE	R	R	R	R	nd		S		5–10	nd	nd	nd

Immunofluorescence tests (IF) and CIE were repeated several times with 5 different sera containing anti-HNA antibodies. IF tests or CIE were performed on human thyroid cryostatic sections or HLE respectively, after physicochemical treatment.

R Resistant; r Sensitive; wr Weak sensitive (partially inactivated but not abolished); S soluble; I insoluble; nd Not done.

Table 3. Effect of enzymatic treatment on HNA

Technique	DNase (100–200 μg/ml)	RNase (100–200/μg/ml)	Neuraminidase (0·01–1·00 U/ml)	Pronase (0·5–20·0 μg/ml)	Trypsin (0·5–20·0 μg/ml)
IF	r	R	wr	wr	wr
CIE	r	R	wr	r	r

Indications as in Table 2.

lines. Comparison with reference sera and sera demonstrating positive reactions chiefly with HNA, showed that: (1) five sera exhibited specificity for the latter: merely with the latter in two samples; associated with anti-RNP or anti-Sm in two and one samples respectively; (2) four hard anti-SSA/Ro antibodies alone (two samples) or associated with SSB/La antibodies (two samples). Two sera had unidentified precipitating antibodies against HLE. Three sera had low titre anti-dsDNA antibodies and two had anti-gastric mucosa or anti-mitochondrial antibodies. Anti-gammaglobulin factors were found in three sera. Age, sex and diagnosis of patients with antibodies to HNA masked by other ANA are reported in Table 4.

DISCUSSION

In the present study, we have demonstrated the presence of autoantibodies reacting chiefly with nuclear antigen(s) of human origin in a limited number of patients. Some samples also gave weak positive reactions with nuclei of other species, but these antibodies were easily absorbed with rat liver, rabbit thymus and horse lung acetone powders. Such sera may contain two or more antinuclear specificities, one or more of which are not directed against antigenic material chiefly represented in human nuclei. However we cannot exclude a low cross reactivity between human and non human nuclear antigens.

A restricted reactivity to human nuclear autoantigen(s) was demonstrated for anti-SSA/Ro antibodies (Harmon *et al.*, 1984) and for autoantibodies directed against nuclear mitotic apparatus (Price *et al.*, 1984). However, anti-SSA/Ro antibodies are also able to react with cytoplasmic antigens of human cells (Alspaugh & Tan, 1975; Maddison, Provost & Reichlin, 1981) and were

Table 4. Clinical data and some serological findings of 14 patients in whose sera anti-HNA activity was shown after absorption with rat liver acetone powder

Case no.	Sex	Age	Clinical diagnosis	ANA	anti-dsDNA	Other autoantibodies	CIE		Remarks
							HLE	RTE	
1	F	44	Sicca Syndrome	320	Neg.	IgM RF	anti-HNA	Neg.	Nephritis
2	F	51	CAH	1280	40	—	anti-HNA	Neg.	—
3	F	59	Rheumatoid Arthritis	640	Neg.	anti gastric parietal cell	anti-HNA	anti-RNP	—
4	F	20	SLE	2560	20	—	anti-HNA	anti-RNP	—
5	F	30	SLE	1280	80	IgM RF	anti-HNA	anti-Sm	Sicca Syndrome
6	F	32	SLE	40	Neg.	—	anti-SSA/Ro	Neg.	—
7	F	69	Rheumatoid Arthritis	1280	Neg.	—	anti-SSA/Ro	Neg.	—
8	F	31	SLE	40	Neg.	—	anti-SSA/Ro	anti-SSB/La	—
9	F	32	Glomerulonephritis	160	Neg.	—	anti-SSA/Ro	anti-SSB/La	Raynaud's phenomenon
10	F	40	SLE	40	Neg.	—	UPL	Neg.	—
11	F	68	Rheumatoid Arthritis	160	Neg.	—	UPL	Neg.	Pleuritis
12	F	34	SLE	20	Neg.	anti-SMA, AMA	Neg.	Neg.	Arthritis
13	F	41	Healthy*	640	Neg.	—	Neg.	Neg.	—
14	F	60	CAH	160	Neg.	IgM RF	Neg.	Neg.	Polymyositis Diabetes Mellitus

The reciprocal of the ANA titre on rat liver cryostatic sections is reported. After absorption, the fluorescence on rat liver nuclei disappeared while that on human thyroid nuclei remained. RTE = rabbit thymus extract; UPL Unidentified precipitin line.

* Two sisters with PBC.

Other indications as in Table 1.

demonstrated to be ribonucleoprotein (Lerner *et al.*, 1981; McNeilage, Whittingham & Mackay, 1984). Furthermore, we were unable to find identities between our sera and SSA/Ro reference sera by CIE. In addition, the IF pattern obtained with SSA/Ro was quite different from that shown by the antibodies identified by us. The properties of our antigen(s) and antibodies are also different from those shown by the NuMA antigen and antibody system.

We can therefore conclude that the antibodies we have identified possess a specificity never described previously. The identification of such autoantibodies was possible by the systematic combined use of human and rat tissues in routine tests for autoimmunity.

The prevalence of sera containing only antibodies reacting chiefly with human nuclear antigens is very low. However, by absorption assays and by comparison with reference sera, we were able to find this specificity more frequently, in association with other antinuclear antibodies.

In seven out of 23 sera containing antibodies directed to human nuclei we were unable to obtain precipitin lines by CIE against HLE. This may be due to a defect of the antigenic material in HLE extracts or else to the presence in such samples of antibodies directed to other nuclear antigen(s). Our data are still inconclusive on this point.

The great majority of patients with antibodies to HNA not associated with other antinuclear antibodies were females with mild connective tissue disease. None of them had SLE. Only one patient, in whose serum anti-HNA was associated with a non identified antinuclear antibody, fulfilled the ARA revised criteria (Tan *et al.*, 1982) for the diagnosis of SLE. Most patients were affected by cutaneous lupus, undefined connective tissue disease, sicca syndrome, rheumatoid arthritis or idiopathic interstitial pneumonia. Diagnosis of chronic hepatitis, polymyositis-dermatomyositis or autoimmune thrombocytopenic purpura was less frequent. All patients with anti-HNA antibodies had mild symptoms, the illness presenting a benign course.

However, when anti-HNA activity was masked by the presence of other ANA, SLE was more frequently found and the prognosis of the illness worse. The presence of anti-HNA antibodies in the serum therefore appears to be associated with a better prognosis, but these antibodies do not seem capable of improving the course of the illness when other ANA are present. It should be pointed out that only one patient with anti-HNA and anti-SSA/Ro antibodies had circulating immune complexes, detected by the C1qBA method (Zubler *et al.*, 1976), while 4 out of 9 patients with anti-HNA antibodies masked by other ANA specificities had circulating immune complexes (data not shown).

On the basis of the results of the physicochemical and enzymatic treatments we can state that the antigen(s) identified by our autoantibodies is a DNA (glyco)-protein complex extractable with saline solutions, resistant to 56°C for 6 h and stable at pH values ranging from 3 to 10.

We have no data either on the possible specific function of this nuclear material or on its precise distribution within the nuclei. Further studies in this field will probably be useful in order to enhance our knowledge on the molecular biology of human nuclear function.

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