Evidence of autoantibodies to cell membrane associated DNA (cultured lymphocytes): a new specific marker for rapid identification of systemic lupus erythematosus

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Abstract

Objective—Autoantibodies to cell membrane associated DNA are described in systemic lupus erythematosus (SLE). The specificity of these antibodies differ from antibodies to nuclear DNA.

Methods—Using indirect immunofluorescence, a specific IgG was detected giving a characteristic pattern of continuous peripheral membrane fluorescence on cultured B-lymphocytes.

Results—This pattern was observed in 53 of 80 serum samples of SLE patients but absent in the serum samples of the control populations: 15 rheumatoid arthritis, 38 ankylosing spondylarthritis, 17 non-inflammatory osteopenic patients, and 224 blood donors. In 34 Sjögren syndrome's patients one only showed a positive test. The cmDNA specificity of these antibodies was confirmed by pattern extinction with DNase but not RNase or protease pre-treatment of the cells. IgG to cmDNA, separated by absorption/elution from purified cmDNA immobilised on DEAE-nitrocellulose reproduced the immunofluorescence pattern pictures. Extensive serum depletion of anti-double strand or single strand DNA antibodies by absorption to cellulose bound ds- or ss-DNA affected marginally the pericellular fluorescence revealing some minor cross reactivity with nuclear DNA. Moreover, in SLE patients without detectable antibody to ds-DNA, pericellular fluorescence could be visible.

Conclusion—This novel rapid immunofluorescence method may serve as an identification test of SLE patients. Given its positive (97.1%) and negative (92.9%) predictive value, sensitivity (66%) and specificity (99.5%), it improves on other diagnostic tests such as the detection of antibodies to Sm.

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sponse to treatment.²⁻⁶ Antibodies to ss-DNA have also been described in autoimmune diseases, although the relevance of these antibodies to disease remains controversial.^{1-3,6}

While for the most part antigenic DNA is localised to the cell nucleus, and presumably becomes available as a result of cell death, evidence has been presented for the existence of another species of DNA that is associated with the outer leaflet of the plasma membrane.⁷⁻¹³ This cell membrane associated DNA, was first described in 1973 by Meinke and coworkers.¹¹ It originates at the cell nucleus and is expressed at the cell membrane as a 17 kb polynucleotide chain on the surface of the B lymphocytes, monocytes, and their transformed analogues. Specific binding of serum autoantibodies to this cmDNA has been debated.⁸⁻¹⁴ Antibodies to cmDNA associated proteins like histones have been described.¹⁵⁻¹⁹ On the other hand, antibodies to a 30 kDa protein that might serve as a DNA receptor for the cell membrane associated DNA have been reported.¹⁵⁻²⁰⁻²⁵ Furthermore, IgG antibodies to DNA from serum samples of SLE patients were found to bind to the cell surface of mononuclear cells.⁸⁻¹⁴⁻¹⁹⁻²⁶

Whether IgG antibodies from SLE patients have antigenic specificity to cmDNA that is distinguishable from that of nuclear DNA has not been investigated.

In this study, we demonstrate that cmDNA can be a specific target for IgG autoantibodies from SLE patients. We also demonstrate that these antibodies contain a non-overlapping specificity, unrelated to ds- or ss-DNA, and that the binding to the cell surface is not based on immune complex formation and Fc receptor interactions. The binding of antibody to cmDNA can be visualised as a highly reproducible pericellular immunofluorescent pattern on a human B-cell transformed cell line. This characteristic pattern, which is very sensitive and highly specific, can serve as a new marker for SLE.

Methods

PATIENTS AND CONTROLS

Serum samples were from SLE patients (n=80) routinely collected as available at the outpatient clinic of the Internal Medicine Department and the Rheumatology Department of the Clinique Cesar DePaepe and Center Hospitalier Universitaire Brugmann (Free University of Brussels). This population was characterised by: a women/men ratio of 4.3; an age range from 25 to 85 years old, median 34. SLE

High titres of serum antibodies to nuclear DNA are considered an important characteristic of systemic autoimmune diseases.¹⁻⁶ Increased levels of autoantibodies to ds-DNA are closely associated with the disease activity in most cases of systemic lupus erythematosus (SLE). The presence of these antibodies defines the clinical subset of the disease and provides information for prognosis and re-

diagnosis fulfilled four or more of the 1982 revised criteria of the American Rheumatism Association (ARA).⁶

Serum samples were also obtained from patients with Sjögren's syndrome meeting the criteria from Kaplan^{26a} and not presenting up to four criteria for SLE (n=34), from patients with rheumatoid arthritis following the criteria of ARA 1987, (n=15), patients with osteopenia (n=17), and patients with ankylosing spondylarthritis (n=38). These serum samples were also selected as available.

Healthy control donor serum samples were provided by blood donors attending the blood bank, Centre Albert Hustin - CHU St Pierre (Brussels, Belgium) (n=224). The healthy donor population was characterised by: a women/men ratio of 1.6; an age range from 18 to 62 years old, median 42. All serum samples were kept at -20°C until use.

B LYMPHOMA CELL LINE WIL2 NS

The B lymphoma cell line Wil2 NS was obtained from ICN Flow laboratories (ECACC no 90112121). Cultures were maintained at 37°C and 5% carbon dioxide in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, and 40 µg/ml of gentamycin. Log phase cultures at a cell density of $1-1.2 \times 10^6$ cells/ml, exhibiting typan blue exclusion of greater than 95% were harvested by centrifugation and used for experiments. RPMI 1640 medium, fetal calf serum, L-glutamine, gentamycin, Hanks's balanced salt solution were obtained from GIBCO-Life Technologies/Gaithersburg USA.

INDIRECT IMMUNOFLUORESCENCE ON WIL2 CELLS

Immunofluorescence on methanol fixed cells

Wil2 cells were washed twice with Hanks's solution, resuspended at 0.5×10^6 /ml, and subsequently spotted on chambered glass slides divided into 20 µl wells. After drying at 37°C, and fixing for three minutes in methanol at room temperature, the cells were washed once in phosphate buffered saline (PBS, 10 mM pH 7.4). Incubation was then undertaken in the presence of goat IgG (Organon Technica; 6 µg/well, 20 µl PBS) to saturate the Fc-gamma receptors on the cell membrane to avoid the further non-specific binding of seric or fluorescent conjugated IgG by their Fc portion. The cells were washed with PBS, and different dilutions of patient or control serum samples in PBS-TWEEN 20 (0.05% v/v) (20 µl/well) were added for 25 minutes at room temperature followed by a wash with PBS. Aliquots of thawed serum dilution samples were heat inactivated at 56°C for 30 minutes before use. In some experiments a purified IgG fraction from patient serum was used. After the PBS wash, fluorescein conjugated goat antihuman IgG (Kallestadt) was added for 30 minutes and then washed twice in PBS containing Evans blue as counterstain. The slides were mounted in glycerin/PBS (2:1), pH 9, and the fluorescence was visualised by an ultraviolet immersion microscope (Leitz Orthoplan) (magnification = 10×40).

Immunofluorescence on non-fixed cells

All reactions were at 4°C. Briefly, cells (1×10^6) in 1 ml of culture medium were placed in plastic tubes (5 ml) and washed with PBS containing 10 mM EDTA and 0.01% Na azide (PBS EA) by centrifugation ($500 \times g$, 10 min). The pellet was resuspended with 0.9 ml of the same buffer supplemented with goat IgG (300 µg/ml) and incubated for 30 minutes. After two washes, cells were incubated with serum or purified IgG at different dilutions in PBS TWEEN (0.05%) for 30 minutes. This was followed by two additional washes and incubation with a fluorescein conjugated goat antihuman IgG (Kallestadt). After four more washes, the cell pellet was resuspended in PBS EA containing Evans blue as a counterstain. Ten microlitres of the cell suspension was placed onto a glass slide, covered, and visualised by ultraviolet microscopy.

Interpretation of immunofluorescence patterns

The immunofluorescence pattern of the cytoplasmic membrane were scored as follows: "0" for the absence of any membrane fluorescence, corresponding to a negative result; "1" for a fluorescence with a punctuate cell membrane appearance; and "2" for a fluorescence displaying a pericellular (peripheral membrane staining) continuous ring.

ENZYMATIC PRE-TREATMENT OF CELLS

After methanol fixation of the slides (see above), cells were incubated with DNase RQ1, RNase free (Promega), 20 µg in 20 µl of PBS for 16 hours at room temperature. The RNase treatment (RNase ONE Promega; 2 µg in 20 µl of PBS) was in parallel but at 37°C. Pronase (Sigma, 125 ng in 25 µl of PBS) or trypsin (Sigma, 3.125 µg in 25 µl of PBS) pretreatment were performed for 30 minutes at 37°C. After washing the slides in PBS (twice), subsequent steps of the procedure were as described above.

DNASE PRE-TREATMENT OF THE DILUTED SERUM SAMPLES

After heat inactivation of serum dilutions (1/40), a 50 µl sample was added to 5 µl of DNase RQ1, (5 µg) for 60 minutes at room temperature. The DNase activity was quenched with 5 µl EDTA (0.5 M). For control 5 µl of enzyme free buffer and 5 µl EDTA (0.5 M) was added to the samples.

ISOLATION OF CMDNA FROM WIL2 CELLS

Cytoplasmic membrane DNA was prepared by the criteria established by Bennett *et al.*⁷ Briefly, Wil2 cells (1×10^9 cells) were divided in two 50 ml plastic tubes and washed twice in Hanks's balanced salt solution and twice in reticulocyte buffered saline (RBS). The pellets obtained were resuspended in 10 ml RBS and 30 µl NP40 (Sigma) was added in each tube (final concentration of 0.3% NP40) followed by a 10 minute incubation at room temperature. This was followed by a centrifugation ($2500 \times g$, 5 min) and the recovered supernatants were brought to a volume of 16.7 ml by the addition of the following to the final

concentrations indicated: EDTA (10 mM), NaCl (1 M), SDS (0.6%) (w/v), and RBS. After mixing by inverting 10 times, the lysates were incubated overnight at 4°C. Lysates were centrifuged (20 000 × g, 30 min) and supernatants were extracted twice with phenol, and once with chloroform: isoamylalcohol (24:1; v/v). Two volumes of cold ethanol were added and incubated overnight at 4°C. The precipitates were centrifuged (20 000 × g, 30 min) and the pellets were resuspended in 0.2 ml TRIS (10 mM)-EDTA (1 mM) buffer. A one hour digestion at 37°C with RNase (1 µl for 30 µl of crude DNA) was followed by purification on a Sephadex G50 spin column (500 × g, 4 min). The recovered DNA was further treated with pronase (10 µl) for one hour at 37°C and purified as above on a Sephadex G50 spin column. Samples were resolved by agarose gel electrophoresis (1%) and showed the presence of a single DNA band of approximately 17 000 bp).

We verified also by an independent technique for isolation of Wil2 membrane^{7 8 10 11} that the DNA containing gradient fraction from Wil2 cells was well associated with another membrane associated product, namely IgM in the present case. The fraction density (1.258 in a 45–60% sucrose gradient) was identical to the density of plasma membrane and was also found to be positive for immunofluorescence with anti-cmDNA antibodies.

ISOLATION OF SERUM IGG FRACTION AND ANTI-CMDNA IGG ANTIBODIES

IgG from selected serum was isolated by affinity chromatography (HiTrap protein G Sepharose, Pharmacia). Antibodies to cmDNA were purified by absorption-elution of IgG fractions on solid phase adsorbed antigen as described: after agarose electrophoresis (1%) of the cmDNA, the resolved 17 kb band was adsorbed onto DEAE nitrocellulose membranes (Schleicher and Schuell NA45). A saturation of free sites onto the nitrocellulose is done by immersing for 15 minutes in PBS containing 5% (w/v) non-fat dry milk. After three washings in PBS alone, the cmDNA containing nitrocellulose membrane was then immersed in the IgG fraction for 15 minutes. After two five minutes washes in PBS, the bound IgG was eluted with 0.5 M NaCl in 0.01 M phosphate buffer by incubation for 15 minutes. Repeated cycles of adsorption and elution were performed.

DETERMINATION OF TITRE OF ANTI-DS- OR SS-DNA IGG

A routine ELISA was performed to determine serum titres of autoantibodies to ds- or ss-DNA. Briefly, purified ds-DNA from calf thymus (Boehringer) digested with exonuclease, RNase, and pronase, was coated on polystyrene microtitre plates (Covalink; NUNC) as the immobilised solid phase. Alternatively, ds-DNA was boiled to obtain single stranded DNA and used in the same manner. Horse serum served as the blocking agent and horseradish peroxidase conjugated rabbit anti-human IgG (DAKO) was used to detect bound

Figure 1 Immunofluorescence patterns on methanol-fixed Wil2 cells. (A) pattern "0" = absence of any significant fluorescence; (B) pattern "1" = punctuate membrane fluorescence; (C) pattern "2" = continuous membrane peripheral fluorescence.

human IgG. The specificity of the tests were determined as follows: (a) extensive cross absorptions of serum on solid phase bound ss and ds DNA demonstrating occasional but non-systematic minor cross reactivity; (b) correspondence with *Crithidia luciliae* fluorescence tests; (c) large series of double determinations in parallel displaying no correlation between titres in ss or ds DNA antibody titres, whatever the low or the high range levels; (d) interlaboratories (n=50) comparisons in periodic quality control routine procedures, 3 times/year (Belgian External Quality Assessment of Scheme).

SERUM DEPLETION OF ANTIBODIES TO DS- OR SS-DNA

SLE patient serum that was positive for the pericellular fluorescent pattern ("2") was depleted of antibodies to ds- or ss-DNA by passage through affinity columns of ds-DNA or ss-DNA cellulose (Sigma). A serum volume of 0.5 ml was passed on a column bed volume of 1.5 ml. The volume of the serum effluent was

Figure 2 Comparative histogram. Distribution of fluorescence patterns in SLE compared with normal serum samples at three different dilutions: 1/2, 1/20, 1/40.

adjusted to 20 ml to obtain a 1/40 final dilution. The collected effluents were tested for antibodies to cmDNA detection and to ds- or ss-DNA using our standard ELISA.

Results

IMMUNOFLUORESCENCE PATTERNS ON METHANOL FIXED WIL2 CELLS

The binding of IgG from patient or control serum samples on methanol fixed Wil2 cells produced three distinct immunofluorescence patterns. These patterns were classified as follows: "0" for the absence of any significant fluorescence, "1" for a punctuate membrane fluorescence, and "2" for a continuous peripheral membrane (pericellular) fluorescence (fig 1).

Figure 2 is a comparative histogram of the relative distribution of the three immunofluorescent patterns obtained for three dilutions of serum (1/2, 1/20, 1/40) from SLE and healthy populations. Pattern "2" was exclusively associated with a first cohort of 50 SLE patients at any of the used dilutions. Indeed, 66% (33 of 50) of the samples from this patient population displayed the continuous pattern "2" at a dilution of 1/40, rising to 69% at 1/20, and 78% at 1/2. Serum from the other tested populations did not display the "2" pattern at any of the dilutions tested above that of 1/2, except for one of 34 Sjögren's patients (table 1). Ten per cent (5 of 50) of the SLE patients serum samples and none of the healthy control samples showed a punctuate pattern "1" at a dilution of 1/40. However, 12% to 17% of the SLE patients serum samples showed also a "1" pattern at dilutions of 1/20 and 1/2, respectively (data not shown). However, this did not seem to be an SLE specific marker as 7% to 20% of the controls showed a similar punctuate pattern at the respective dilutions.

To determine the specificity of the pericellular fluorescence pattern "2" as a marker of SLE we analysed the fluorescence pattern on Wil2 cells of serum from patients with Sjögren's syndrome, rheumatoid arthritis, ankylosing spondylarthritis, and osteopenia at a serum dilution of 1/40, compared with an extended series of 80 SLE patients. The results shown in table 1 clearly demonstrate that the "2" pattern is specifically associated with the SLE diagnosis. The only exception found was in one of 34 serum samples (3%) of Sjögren's patients,

Table 1 Frequency of the circular fluorescent pattern ("2") in different patient populations (serum dilution 1/40). Sensitivity and specificity of the immunofluorescence assay

	Negative pattern (0 or 1)	Positive pattern (2)	Cohort
Sensitivity*			
SLE	27 (33)‡	53 (66)	80
Sjögren's syndrome	33 (97)	1 (3)	34
Ankylosing spondylarthritis	38 (100)	0 (0)	38
Rheumatoid arthritis	15 (100)	0 (0)	15
Osteopenia	17 (100)	0 (0)	17
Healthy controls	224 (100)	0 (0)	224
Specificity† versus			
Healthy controls	224 (100)		
Osteopenia	17 (100)		

*Sensitivity is defined as the ratio of diseased patients correctly classified by the assay, divided by the total number of diseased patients, expressed as a percentage. †Specificity is defined as the ratio of non-diseased patients correctly classified by the assay, divided by the total number of non-diseased patients, expressed as a percentage. ‡Number (percentage). Fluorescence pattern scores: 0 = no significant picture; 1 = disseminated points on the membrane; 2 = continuous ring around the cells.

which also showed the pericellular fluorescence pattern, all other tested samples were negative for the "2" pattern. The sensitivity and specificity of this immunofluorescence assay were calculated showing that: 66% of SLE patients were detected and 100% of the healthy control population was correctly classified by the absence of the immunofluorescence pattern "2". This remained true for lower dilutions (data not shown). The assay was quite sensitive by correctly predicting 66% of the SLE population had SLE regardless of the individual treatment protocol and progression of disease. In other populations tested, the assay shows almost no sensitivity with only one of 34 (3%) serum samples from Sjögren's patients and 0% of other patients serum samples showing a positive picture at a serum dilution of 1/40. The positive and negative predictive value of our test for SLE are respectively 97.1 and 92.9%.

The reproducibility of the test was determined as follows: 100 selected samples displaying the following spectrum of responses: 39 pattern "0", 12 pattern "1", and 49 pattern "2". These samples were tested blind and independently by three experimentators.

Concordance of the results was: 98% in interassay testing, on the same lot of slides, and 97% on a different lot of slides. The observed discordant readings appeared between patterns "0" and "1" and never on pattern "2", where 100% concordance was found. Moreover, for the purpose of this study, all reported cases were read blind at least on two occasions.

IMMUNOFLUORESCENCE PATTERNS ON NON-FIXED WIL2 CELLS

To avoid permeation of the cells to antibodies, and the potential consecutive revelation of anti-nuclear activity, the cells were fixed with methanol only (without acetone). To control for membrane permeability, protein aggregation, or binding to intracellular epitopes caused by methanol fixation of Wil2 cells, we analysed the immunofluorescence patterns of control and patient serum samples of IgG on non-fixed Wil2 cells in parallel with methanol fixed cells.

Table 2 Immunofluorescence patterns of purified IgG and anti-cmDNA antibodies on different cell substrates

Tested material	Immunofluorescence patterns*	
	on fixed cells	on non-fixed cells
Controls (n=5)		
serums	0†	0
IgG fractions	0	0
SLE patients (n=6)		
1 serum	2	2
IgG fraction	2	2
anti-cmDNA IgG	2	2
2 serum	2	2
IgG fraction	2	2
anti-cmDNA IgG	2	2
3 serum	2	2
IgG fraction	2	2
anti-cmDNA IgG	2+1	2+1
4 serum	2	2
IgG fraction	2	2+1
anti-cmDNA IgG	2+1	2
5 serum	2	2
IgG fraction	1	2+1
anti-cmDNA IgG	1	2+1
6 serum	2	2
IgG fraction	2	1

*Confirmed on three different assays. †Fluorescence pattern scores: 0 = no significant picture; 1 = disseminated points on the membrane; 2 = continuous ring around the cells.

The results summarised in table 2 show that in three experiments the fluorescence patterns of sera IgG from controls and patients showed identical fluorescent patterns to that observed in methanol fixed cells (fig 1, table 1). Nevertheless, the pericellular fluorescence pattern "2" detected on fixed cells was less consistent on non-fixed cells with the appearance of an arc-like pattern instead of a diffuse continuous peripheral membrane ring.

We also tested purified IgG fractions and anti-cmDNA on both types of cells: the IgG fractions of six serum samples that gave the "2" pattern were obtained by affinity chromatography on protein G-sepharose. The fluorescence pattern on fixed Wil2 cells was identical to that observed with whole serum samples for five of the six IgG fractions tested (table 2). The one exception now displayed an immunofluorescent pattern similar to the "1" pattern. Identical results were obtained on non-fixed cells, however, a second IgG fraction displayed an immunofluorescent "1" pattern. It should be noted that the two IgG fractions that displayed a pattern, which differed from that displayed by the whole sample, had the lowest concentration of IgG. Thus it is possible that the pericellular fluorescent pattern "2" requires a threshold concentration of antibody that was not met by the purification of only the IgG fraction from these two samples.

Antibodies directed to cmDNA were purified from five IgG fraction by affinity adsorption and their immunofluorescence pattern tested on fixed and non-fixed cells as shown in table 2. The immunofluorescence pattern of the purified antibody on fixed cells was identical to that of the originating serum for four of the five antibodies tested. The exception again displayed an immunofluorescent "1" pattern and was also found to have the lowest IgG concentration suggesting the requirement of a threshold concentration of antibody to cmDNA to display the typical immunofluores-

Table 3 Influence of DNase and RNase treatment of the cells on fluorescence pattern

Populations	IF pattern	Cell treatments		
		No	DNase	RNase
SLE patients (n=15)	1	1.4%*	0.2	0.1
		3.3†	0.6	0.4
	2	77.8%	2.4	74.8
		5.7	2.9	13.4
Controls (n=15)	1	1.3%	0.8	0.7
		2.4	1.1	1.2
	2	2.0%	5.7	5.3
		2.5	5.9	6.6

*Mean percentage of fluorescent cells determined on 100 individual cells per tested serum with the same pattern (dilution 1/40). †Standard deviation of the mean percentage.

cent "2" pattern seen from serum of SLE patients. Interestingly, in two other cases the purified antibody to cmDNA showed an immunofluorescent "1" pattern in addition to the expected "2" pattern. This dual immunofluorescent pattern appeared when non-fixed cells were used in the assay. Nevertheless, with non-fixed cells all the purified antibodies from the five serum samples showed the immunofluorescent "2" pattern that was observed with the originating serum.

Other cells were tested for the presence of cmDNA through the immunofluorescent test with appropriated reference serum samples or purified antibodies. Fluorescent pattern "2" appeared also on cultured cell lines: Daudi cells (EBV transformed human B cell line lacking MHCII expression), mouse Sp20 (non-secreting myeloma cells), but not on Molt3 (transformed human T cell line). Bone marrow cells from a patient with lymphoblastoid leukaemia (early B cells) was also positive. The same pattern "2", presumed to indicate the existence of similar antigen is thus visible on human and mouse cells sharing a B cell lineage origin, a common characteristic already reported for the presence of cmDNA.

EFFECT OF DNASE TREATMENT ON WIL2 CELLS AND SERUM SAMPLES FROM SLE PATIENTS

We investigated the possibility that the DNA localised to the outer cell membrane is itself the antigen targeted for antibody binding. To test this hypothesis we pre-treated Wil2 cells with DNase or RNase and analysed the immunofluorescence patterns obtained with treated compared with non-treated cells. We screened and scored the immunofluorescence pattern of 100 individual cells for each experiment. Table 3 summarises the findings as the per cent of cells that displayed an immunofluorescent "1" or "2" pattern. Of the fifteen SLE samples tested, in all cases DNase pre-treatment, but not RNase pre-treatment, effectively abolished the fluorescence pattern "2". For control samples, neither DNase or RNase treatment had any effect on the fluorescence pattern ("0") observed. Furthermore, the overnight treatment with DNase or RNase had no obvious effect on the cell membrane as monitored by the presence of CD19 (a common B cell lineage membrane surface marker) on these cells.

To exclude the possibility that the patient serum contained circulating DNA or DNA-IgG immune complexes that might react with

Table 4 Comparison between IF on Wil2 and anti ds- or -ss DNA antibodies. Eighty serum samples were classified according to the fluorescence pattern on Wil 2 cells: "2" pattern or other pattern ("1" or "0"). The medians of titres in anti ds- or ss- DNA antibodies obtained in two independent ELISA were indicated in relation with each fluorescent pattern

Antibody specificity		Titres of antibody according to immunofluorescence patterns	
		0 or 1	2
-dsDNA	median	27*	65†
	range	1-61	0-5281
	mean (SD)	28 (18)	224 (734)
-ss DNA	median	56	145‡
	range	2-201	0-400
	mean (SD)	64 (50)	193 (151)

*Arbitrary units. "0" or "1" versus "2" (Mann-Whitney): †p=0.0004 ‡p=0.0013.

the cmDNA by hybridisation or other interactions, we pretreated the samples of 12 SLE patients with DNase. All samples had previously shown an immunofluorescence "2" pattern. After DNase treatment all samples exhibited the same pattern although in two cases the fluorescence intensity was decreased.

The antigen recognised in pattern "1" is not yet defined. Pattern "1" appeared as quite unstable and could result from structures absorbed on the cell membrane. Indeed, mild acidic washing of the cells destroyed pattern "1" identification, without any effect on pattern "2" recognition. Moreover, mild trypsin or pronase digestion of the fixed cells abolished always pattern "1" expression, while this was not reduced by RNase or DNase pretreatment.

ANALYSIS OF A POSSIBLE CORRELATION OF IMMUNOFLUORESCENCE ON WIL2 CELLS AND THE SERUM CONTENT OF ANTIBODIES TO DS- AND SS-DNA

The antibodies to ds- and to ss-DNA were measured in two independent ELISAs for the SLE patients. Among 80 samples of SLE patients, 40 had a anti ds-DNA level below the threshold of 40 AU/ml (arbitrary units per ml), a level not specifically associated, in our system, with SLE. Nevertheless, 18 of these samples had a positive pattern on Wil2 cells, indicating again a qualitative dissociation of the two tests. Considering the 40 samples with high titres of anti ds-DNA IgG antibodies, (> 40 AU/ml), 33 of them had a positive pattern "2" of immunofluorescence on Wil2 cells. This increased proportion was significant ($p < 0.001$, χ^2 test).

The median of the titres was compared for 80 SLE patients subdivided into two categories according to the presence of immunofluorescent pattern "2" ($n = 53$) or its absence ($n = 27$). Table 4 summarises the statistical parameters. The presence of fluorescent pattern "2" is clearly associated with significant increased levels of ds-DNA and ss-DNA IgG antibodies. It should be noted that values displayed for both specificities have a broad overlapping range including non-detectable values for these anti nuclear DNA antibodies.

Table 5 Comparison between IF patterns on Wil2 cells and the titres of anti-nuclear antibodies determined on HEp2 cells. Results of 80 LED serum samples were cross compared according to the fluorescence pattern observed on Wil 2 cells (patterns "2" v "0/1") and their respective titres (last dilution for a visible picture) measured on HEp2 cells. These were graded in categories defined as: negative below the serum dilution of 1/80, then from 1/80 to 1/320, from 1/640 to 1/1280 and >1280

Dilutions on HEp2	Pattern on Wil2		Total	
	0 or 1	2		
<80	1	1	2	
80-320	8	3	11	
640-1280	12	13	25	
>1280	8	34	42	χ^2 13.37
Total	29	51	80	$p=0.0039$

DEPLETION OF ANTIBODIES TO DS- OR SS-DNA FROM SLE SERA AND INCUBATION ON WIL2 CELLS
Six SLE serum samples with an immunofluorescent "2" pattern were depleted of antibodies to ds- and ss-DNA by affinity depletion chromatography with ds-DNA- or ss-DNA-cellulose.

The samples had an initial content of ds- or ss-DNA IgG ranging from 40 to over 400 U/ml, as determined by ELISA. The effect of depletion was analysed by ELISA with resulting titres below the detection limit of the assay (<10 U). The immunofluorescent tests on Wil2 cells were performed in parallel with non-depleted serum samples. Depletion of antibodies to ds-DNA had no effect on the immunofluorescent pattern observed in all the cases. Depletion of antibodies to ss-DNA also failed to modify the fluorescence pattern, although in three cases the fluorescence intensity was diminished.

By contrast, absorption/elution of SLE IgG on Wil2 surface antigens before anti ds- or ss-DNA testing confirmed the specificity of the retained antibodies. Some minor cross reactions between cmDNA and ss- or ds- DNA for the same antibodies' preparation were occasionally visible, suggesting that among anti-cmDNA IgG, some minor part could indeed cross react with nuclear DNA. Other fractions are expressing a restricted reactivity exclusive for DNA from membrane origin.

COMPARISON OF IMMUNOFLUORESCENT PATTERNS ON WIL2 CELLS AND THE TITRES OF ANTINUCLEAR ANTIBODIES DETERMINED ON HEP2 CELLS

Table 5 summarises the cross comparison between the results of 80 SLE serum samples according to the immunofluorescent pattern observed on Wil2 cells and titres of antinuclear antibodies determined on HEp2 cells. These were graded into four categories of dilution ranges, allowing an immunofluorescent picture detection, of increasing value: < 80; 80-320; 640-1280; > 1280.

Wil2 positive fluorescent pattern "2" was visible in the four quantitative categories of antinuclear IgG titres, even when the latter would have been considered as negative (one of two subjects), indicating already a lack of strict correspondence between the two tests.

With increasing titres of antinuclear IgG, the proportion of samples exhibiting a positive pattern on Wil2 cells was increasing significantly ($p < 0.005$, χ^2 test).

Table 6 Comparison between the IF patterns on Wil2 and those on anti-nuclear antibodies observed on HEp2 cells. Results from 80 LED serum samples were cross compared according to their fluorescence pattern on Wil 2 cells ("0/1" or "2"). The picture patterns on HEp2 cells were scored as follows: homogeneous, speckled, combined homogeneous and speckled, "other" for miscellaneous variants and negative for non-significant fluorescence

	Patterns on Wil2 cells		Total	
	0	2		
IF pattern on HEp2 cells				
homogeneous	17	25	42	
speckled	5	20	25	
homogeneous and speckled	4	2	6	
other	2	3	5	
negative	1	1	2	χ^2 5.77
total	29	51	80	p=0.2164

COMPARISON OF IMMUNOFLUORESCENT PATTERNS ON WIL2 AND HEP2 CELLS

Table 6 compares the patterns of immunofluorescent pictures detected on HEp2 cells and the corresponding result on Wil2 cells. No specific immunofluorescent picture on HEp2 cells was associated with any Wil2 pattern ($p > 0.2$, χ^2 test). The most frequent patterns (homogeneous and speckled pictures) were not even significantly discriminant among subjects displaying the Wil2 pattern "2".

Discussion

There is accumulating evidence that DNA is found in association with the cell membrane^{7 10-13 27} in a form bound to a 30 kDa protein acting as a receptor.^{9 14 20-22 27} This study supports that membrane associated DNA molecules (cmDNA) are specific targets for autoantibodies found in SLE patients.⁸

Firstly, the use of a human B cell lymphoma cell line, Wil2 NS, has clearly defined two types of fluorescent patterns associated with different patient populations.

This immunofluorescent assay allowed the recognition of the SLE patient population by the detection of an immunofluorescent pattern consisting of a continuous ring of fluorescence around the periphery of the cells. This pattern was not observed for a population of 224 healthy controls (blood donors), nor in other control disease groups at a serum dilution of 1/40 with one exception of a patient with Sjögren's syndrome. This latter case should not be considered as an argument against the specificity of this assay for SLE because some Sjögren's syndrome patients could later develop SLE. Our findings clearly demonstrate that this immunofluorescent pattern is specific for SLE (99.5%) and is quite sensitive as an assay for SLE with 66% of the diagnosed SLE patients showing a positive fluorescent pattern "2" under conditions where 50% of the SLE patients remained below the threshold level for the conventional anti-nuclear DNA testing criteria. Thus we can conclude from our cumulative analysis that the antigenic specificity of the antibodies that define the immunofluorescent "2" pattern have little or no correlation with antibodies directed to nuclear DNA. A second pattern of punctuate fluorescence "1" was found in 10% of SLE, 5% of Sjögren, and 13%

of ankylosing spondylarthritis patients serum samples at a dilution of 1/40. Rheumatoid arthritis, osteopenia, and healthy control serum samples were all negative. Therefore this pattern is much less discriminating and much more unstable but defines a subset of the patient population with autoimmune disease.

The characterisation of the antigenic target of the autoantibodies that define the SLE population suggests that the target is a DNA moiety localised to the outer layer of the cell membrane. Our assays ruled out that the antigenic specificity is to the conventional nuclear DNA. Purification of the cmDNA and treatment with pronase followed by binding of the antibody to the cmDNA suggests that a DNA associated protein is not a likely antigen.

Moreover, these purified antibodies reproduced the fluorescent pattern observed with the whole serum sample. Furthermore, DNase, but not RNase or proteases treatment of the cells abolished the immunofluorescent marker pattern for SLE for the purified antibodies. We also excluded the possibility of an extracellular origin for the recognised DNA, as passively transferred from the tested samples, by pre-treatment of positive serum samples with DNase. This had no effect on the resulting fluorescent pattern. The type of autoantibody described in this study differs from other described autoantibodies to B lymphocyte. Indeed, others have reported the binding of antibodies to histones that bind to the membrane of B lymphocytes.^{17 23-25}

Furthermore, the presence of autoantibodies directed to a membrane 30 kDa protein that acts as a DNA receptor^{14 15 20 22 27} has also been described. Our results exclude these possibilities and thus all our evidence points to the antigenic nature of the DNA moiety itself. An investigation to define the antigenic specificity of these antibodies is currently in progress.

In a few cases we observed the presence of two distinct immunofluorescent patterns "1" and "2" that resulted from incubation with the purified antibody from a single serum sample. The presence of the additional punctuate pattern of fluorescence "1" could result from traces of contaminant or adsorbed antigen as a minor constituent on the solid phase adsorbent. This would allow the retention of some antibodies unrelated to cmDNA. Alternatively, the "1" pattern that is observed in a subset of the population of autoimmune disease patients may reflect a cross reactive antigenic epitope. The antigenic structure of pattern "1", sensitive to protease and mild acidic washing of the cells could result from the proteic structure absorbed by the cells but remains, as of yet, not identified. Nevertheless, the fact that only some of the purified antibodies to cmDNA demonstrate this dual fluorescence pattern provides suggestive evidence for a polyclonal response elicited by the antigen cmDNA. We consider that all of the results demonstrate the exclusive DNA antigenicity of the cmDNA, which is recognised by the serum of SLE patients.

The fluorescent pattern "2" is negatively correlated with one of the ARA criteria for SLE: criteria 8 (neurological disorders), but

not correlated with any other of them. It is also not associated with the clinical activity of the disease nor with the presence or absence of any treatment with corticosteroids or immunosuppressors.

The source of the DNA bound to the cell membrane remains a controversial issue.²⁷ Rogers and colleagues provide evidence that phytohaemagglutinin stimulated peripheral blood lymphocytes secrete DNA,²⁸ which subsequently becomes bound to the membrane and internalised.²⁷⁻²⁹ It seems probable that the 30 kDa protein acting as DNA receptor is a scavenger for circulating DNA, effecting its removal from the bloodstream.¹⁵⁻²⁷ Bennett and coworkers have shown the presence of a set of autoantibodies in serum samples from SLE patient that are directed to this 30 kDa protein acting as a DNA receptor on T lymphocytes.¹⁵⁻²⁷ The possibility that the cmDNA present on the Wil2 cell membrane could be passively adsorbed nuclear material from non-viable cells seems improbable because of the different antigenic specificity from nuclear DNA and the characteristic size of 17 kbp. It might be expected that passively adsorbed nuclear DNA might maintain its antigenic specificity and be variable in size.

Comparisons with conventional tests have shown a complete dissociation between immunofluorescence pattern 2 on Wil2 cells and ds or ss-DNA IgG. Firstly, titres and ranges of the latter were not related to the pattern "2". The fact that serum samples with the latter pattern had higher ds and ss-DNA IgG titres could represent the patients with a more active disease. Indeed, experiments with specific cross absorptions, in both senses, demonstrated the complete independence of the cmDNA specificity compared with the nuclear DNA one. Similar conclusions were reached comparing conventional anti-nuclear antibody testing on HEp2 cells and the present method. Again no close association could be deduced from either the titre level nor the picture type on HEp2 cells and pattern "2" on Wil2 cells.

In summary, we demonstrate in this study that IgG of SLE patients binds to cmDNA on Wil2 cells. This membrane DNA found at the surface of the human lymphoblastoid Wil2 cells has also been described on freshly isolated white blood cells.^{7-8,29} It may represent a major immunogen in SLE as shown in our results comparing SLE and healthy serum samples. We have also developed a novel immunofluorescence assay based on distinct pattern recognition that can serve as a sensitive and specific diagnostic marker of SLE as routine screening method. This rapid and easy screening method should be useful in the accurate clinical diagnosis of disease.

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