Specificity of antinuclear and antiphospholipid antibodies in sera of patients with autoimmune lymphoproliferative disease (ALD)

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Abstract

Objective

A human lymphoproliferative syndrome characterized by a defect of the Fas-mediated apoptosis pathway in the absence of a fas gene mutation (Autoimmune Lymphoproliferative Disease) has recently been described and characterized by autoimmune phenomena. The aim of this study was to investigate the presence of antinuclear and antiphospholipid antibodies and to define their specificity in 5 pediatric patients with this syndrome.

Methods

Antinuclear antibodies were investigated by Western Blot and IIF performed under standard as well as apoptotic conditions. The fine specificity of antiphospholipid antibodies was dissected by an ELISA for anti-β2-glycoprotein I, anti-prothrombin, anti-annexin V and anti-protein S antibodies, and by immunostaining on thin layer chromatography plates for antiphospholipid molecule antibodies.

Results

This study showed that the autoantibodies found in these patients targeted a broad spectrum of nuclear antigens which undergo redistribution from the nucleus to the cytoplasm and plasma membrane during the course of the apoptotic process. This reactivity does not comprise known specificities such as anti-extractable nuclear antigens or anti-dsDNA. Antiphospholipid antibodies were also found in these sera. A further characterization of the antiphospholipid antibodies showed the presence of a heterogeneous response with antibodies directed to negatively-charged phospholipids and antibodies targeting coagulation-related proteins (β2-GPI, prothrombin, annexin V) which are considered relevant antigens in the antiphospholipid syndrome.

Conclusions

These results suggest that lack of tolerance due to a defect of Fas-mediated apoptosis allows the survival of B and T clones involved in the antinuclear and antiphospholipid immune responses.

Key words

Fas, apoptosis, autoimmune lymphoproliferative syndrome, antiphospholipid antibodies, antinuclear antibodies.

This study was supported by Telethon, Project w.E. 1170; FISM (Federazione Italiana Sclerosi Multiple).

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Received on April 26, 2002; accepted in revised form on January 10, 2003.

Introduction

It has been postulated that a defect of apoptosis contributes to the generation of different antibodies, including antinuclear (ANA) and antiphospholipid (aPL) antibodies (1, 2). The detection of ANA and their characterization represents an invaluable tool for the diagnosis of connective tissue diseases, while the presence of aPL and/or the lupus anticoagulant is associated with the antiphospholipid antibody syndrome (APS), which is characterized by arterial or venous thrombosis and recurrent miscarriages (3). Investigating the molecular sequence of events leading to apoptosis has highlighted the primary role of the fas gene, which also plays a pivotal role in the maintenance of the homeostasis of the immune response.

The Fas molecule (CD95), which is a transmembrane protein belonging to the TNF receptor superfamily, binds to its specific ligand (FasL) and thereby generates the intracellular signal leading to apoptosis (4). The precise role of Fas in human pathology is still under investigation. A genetic structural defect of Fas has not been commonly found in known autoimmune diseases, but it has been in the few cases of the human autoimmune lymphoproliferative syndrome (ALPS) (5, 6) in analogy with the MRL *lpr/lpr* mouse, the murine model carrying the Fas structural defect (7).

Some of these young patients have clinical autoimmune manifestations such as hemolytic anemia, thrombocytopenia, neutropenia, glomerulonephritis and vasculitis. The broad expansion of CD4/CD8 double negative T cells in the peripheral blood is the most striking immunological feature of this syndrome. More recently, an autoimmune lymphoproliferative condition characterised by a defect of the Fas signalling pathway in the absence of a structural defect of the Fas molecule has also been described (8). This condition does not show the expansion of CD4/CD8 double negative T cells. Thus, a recent classification designated as ALPS type Ia and Ib the disease with mutations of Fas or FasL respectively, and as ALPS-type II or autoimmune lymphoproliferative disease (ALD) the disease in which mutations of these genes are not found (9).

It is well known that ANA are a very heterogeneous population of autoantibodies including antibodies directed to proteins with relevant nuclear functions as well to complex molecular structures including DNA and ribonucleoproteins. The fine specificity of aPL has more recently been dissected with the evidence of antibodies targeting phospholipid (PL)-binding proteins (β2-GPI, prothrombin, annexin V) and antibodies directed to PL *per se*. A number of investigations support the observation that non-bilayer negatively-charged PL and cofactor proteins such as β2-GPI and annexin V are exposed, amongst the nuclear autoantigens, on the surface of cells undergoing apoptosis (2). The exposure and biochemical modification of these clusters of autoantigens on apoptotic membranes may well play a significant role in inducing tolerance or immune response under physiological and pathological conditions, respectively.

Although the precise molecular defect has not been elucidated yet,the autoimmune phenomena found in ALD suggest a possible relationship between the defect of Fas-mediated apoptosis and the production of autoantibodies. On the other hand, studying the specificity of the autoantibodies produced in the course of ALD may contribute to understanding the mechanisms of autoantibody generation in classic autoimmune diseases. In this respect, preliminary evidence has recently been provided that in some human autoimmune diseases an impairment of the Fas-signalling pathway rather than a structural defect of Fas or FasL is found (10). Apoptosis plays a key role in the negative selection of autoreactive cells and in the maintenance of homeostasis in the lymphoid system. Thus, lack of apoptosis at either the central or peripheral level may allow T and B clones to escape from a negative selection and receive the stimula needed for the autoimmune response (11-13).

The aim of this study was to investigate the presence and specificity of ANA and aPL in patients with ALD.

Materials and methods

Patients

As a result of a screening of pediatric patients with autoimmune phenomena and/or lymphoadenopathy in the absence of criteria for a diagnosis of overt autoimmune disease or lymphoma, 5 unrelated patients were selected on the basis of the presence of a defect in the Fas apoptosis pathway. Thrombocytopenia, hemolytic anemia and neutropenia accompanied by the serological detection of ANA were the autoimmune clinical features reported in these patients. We briefly summarize here the main clinical characteristics of each patient studied. Patients 1, 2 and 3 have already been partially reported (8).

Patient 1 was a 24-year-old male seen at the age of 13 for thrombocytopenia, splenomegaly and lymphoadenopathy. Subsequently, he also developed neutropenia.

Patient 2 was a 6-year-old boy hospitalized at the age of 2 for thrombocytopenia and relevant splenomegaly.

Patient 3 was a 13-year-old girl who had persistent fever and substantial splenomegaly at the age of 8 months. Further episodes of fever followed in the next two years. Results of viral and bacterial serology were consistently negative. In the following years, she showed episodes of hemolytic anemia and thrombocytopenia.

Patient 4 was a 13-year-old boy hospitalized for thrombocytopenia at the age of 11. The following year he had an autoimmune hepatitis.

Patient 5 was a 13-year-old girl admitted to our department at the age of 7 for severe hemolytic anemia. She had recurrent episodes of thrombocytopenia or hemolytic anemia. In the last 4 years she had a serious hypogammaglobulinemia.

T cells isolated from the PBL of each patient showed a significant reduction in Fas-induced apoptosis. Cytofluorimetric analysis and a cell survival test provided evidence that T cells from these patients had a decreased apoptotic response when treated with either anti-Fas antibody or ceramide, which is a molecule involved in the Fas signaling pathway downstream from Fas. Mutation analysis of the fas gene, performed by single-strand conformation polymorphism and complementary DNA (cDNA) sequencing, demonstrated the absence of any mutation (8).

Characterization of antiphospholipid antibodies

Sera found to be positive for aPL with a commercially available ELISA (Asserachrom APA, Stago Diagnosica, Italy) underwent a confirmatory test two weeks later and were then further characterized using two highly specific methods: 1) immunostaining of PL on high performance thin layer chromatograpy (HPTLC) plates, and 2) anti-cofactor protein ELISA.

Immunostaining on HPTLC plates. Immunostaining of sera was performed on 2 μg of 5 different PL antigens: cardiolipin (CL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (Sigma Chemical Co., St. Louis, USA) separated by thin-layer chromatography using HPTLC as previously described (14)

Anti-phospholipid binding protein ELISA. Detection of anti-β2-GPI antibodies was performed with a commercially available ELISA folowing the manufacturer's instructions (Diamedix, Miami, USA). Anti-prothrombin, anti-protein S and anti-annexin V antibodies were detected with a previously reported method (15).

Briefly, the anti-prothrombin antibody ELISA was carried out as follows. A solution of purified prothrombin (10 μg/ml) was used to coat (100 μl/well) irradiated polystyrene plates (Nunc Intermed, Roskilde, Denmark). After blocking with PBS-0.1% Tween 20 (PBS-T) containing 3% albumin for 90 min. at room temperature (r.t.), 1:100-diluted (2% albumin-PBS-T) sera were then incubated for 90 min. at r.t. After incubation with conjugated antibody for 90 min. at r.t., the reaction was developed with a substrate solution and read at a wavelength of 405 nm.

Anti-annexin V antibodies were detected by a slight modification of a previously described ELISA method (16), as follows. Irradiated polystyrene plates (Nunc Intermed, Roskilde, Denmark) were coated (100 μl/well) with annexin V (5 μg/ml) (Sigma) in PBS. The plates were blocked by incubation for 2 hr at r.t. with a solution of PBS-T containing 3% albumin. One hundred μl of serum (1:100 diluted in PBS-T-1% albumin) were added and incubated for 1 hr at r.t. After washing 3 times with PBS-T, alkaline phosphatase-conjugated affinity-purified goat anti-human IgG (Fc fragment specific) were added (1:1000 diluted in PBS-T-1% albumin) and incubated for 1 hr at r.t. After washing 3 times, 100 μl of p-nytrophenylphosphate solution were added and the optical density was read at 405 nm when a positive control had reached a value of 1.00 OD. The absorbance of the control wells was subtracted to account for non-specific binding.

A control group of 20 sera from patients with primary APS and from blood donors was also included as representative of these reactivities under pathological and normal conditions, respectively. As previously established, the cut-off value was fixed at the mean OD + 2SD of normal human sera for anti-prothrombin and anti-protein S and at the mean OD + 5SD for anti-annexin V antibodies.

Antinuclear reactivity under standard conditions and after apoptosis induction

Antinuclear reactivity first investigated by IIF on Hep2 cells was further studied on U937 cells under standard conditions and after apoptosis induction.

Cell line culture and induction of apoptosis. A U937 monoblastoid cell line was grown in RPMI 1640 medium with 10% FCS and supplemented with HEPES, penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2 in air and 100% humidity. Apoptosis was induced by culturing the cells in supplemented medium in the absence of FCS (growth factor withdrawal), adding cytokine treatment with TNF-α 50 ng/ml (Sigma) and protein synthesis inhibition with cycloheximide 50 μg/ml (Sigma). The rate of apoptosis was evaluated 90 and 120 min. after culture onset. The viability was also measured in a parallel U937 cell population maintained under standard conditions without additional apoptotic stimulus

(control population). Two methods were used to assess apoptosis:

1) Nuclear morphology and viability. A solution made up of ethidium bromide (100 µg/ml) (Sigma) was used to stain a suspension of unfixed U937 cells (5 x 10^6 cell/ml). Ten µl of the stained cell suspension was placed on a glass slide and covered with a coverslip. Slides were immediately analysed with a fluorescence microscope using a red fluorescence filter. Signs of nuclear condensation and fragmention (apoptotic nuclei) were specifically sought.
2) Cytofluorimetric evaluation of cell DNA fragmentation. DNA fragmentation was studied by propidium iodide staining followed by flow cytometric analysis. Cells were fixed by adding cold 70% ethanol with vigorous stirring and leaving them for 30 min. at 4°C. Cells were then harvested by centrifugation at 200 g for 10 min. at 4°C and resuspended in 800 µl PBS. One hundred µl of RNAse (1 mg/ml) (Type I-A, Sigma) and 100 µl of propidium iodide (400 µg/ml) were added and cells were incubated in the dark at 37°C for 30 min. The suspension was then kept at 4°C in the dark until measured. Fluorescence intensity was analysed on a FACScan flow cytometer (Becton & Dickinson) using the FL2 signal detector for propidium iodide. Seven thousand events were recorded for each sample. The data were analysed using WinMDI software.

Indirect immunofluorescence on apoptotic and viable U937 cells. Sera from the patients (1-5) were tested on cells under standard conditions and after the addition of the apoptotic stimulus using an immunofluorescence method. Briefly, cells were harvested from both cultures and washed once in PBS. Cells were then spun on a slide using a cytospin centrifuge (75 g for 3 min). Slides were left dry, subsequently fixed with methanol on ice for 3 min and kept at –20°C until used. Pre-fixed slides were incubated with sera from patients (1-5) for 45 min at r.t., then washed in PBS. FITC-conjugated anti-human IgG were added for 1 hr at r.t. After repeated washing in PBS with the addition of Blu Evans as fluorescent counterstain, slides were mounted in glycerine and observed using a fluorescence microscope with a FITC outfit (Leitz, Germany). One sample of unfixed cells under standard conditions and one sample after apoptosis induction were directly incubated with sera from the patients and controls (1:20 diluted in PBS-1% albumin) on ice for 45 min. at r.t., then washed in PBS. FITC-conjugated anti-human IgG were added for 1 hr at r.t. After further washing, fluorescence intensity was analysed on a FACScan flow cytometer (Becton & Dickinson) using the FL1 signal detector. Seven thousand events were recorded for each sample.

Total cell protein extract Western blot. The immunoreactivity of sera against a total cell protein extract was investigated using a commercially available Hep2 cell protein extract and following the manufacturer's instructions (Hep2 Marblot Arnika, Milan, Italy). A set of sera from patients with autoimmune diseases including systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS), polymyositis (PM), dermatomyositis (DM), Sjögren's syndrome (SS), overlap of SLE and PSS, and serum from a blood donor were also included as control.

Autoantigens identified by western blot. The reactivity of sera against identified autoantigens was investigated with a commercially available assay using recombinant (SmB, RNP-70k, RNP-A, RNP-C, Ro52, SSB/La, CenpB, Topo-I, Jo1), synthetic (SmD, ribosomal P protein), and natural (Ro60 and Histons) proteins blotted on nylon strips as condensed band. The reactivity of single sera was identified as positive by a comparison with a reference band in order to exclude low, uncertain reactivity and/or non-specific binding (Immunogenetics NV, Gent Belgium). A set of sera from patients with autoimmune diseases including SLE, progressive systemic sclerosis (PSS), polymyositis (PM), Sjögren's syndrome (SS), overlap of SLE and PSS and a set of sera from blood donors were also included as control.

Anti-dsDNA reactivity. Anti-dsDNA reactivity was investigated using an indirect immunofluoresence assay on *Crithidia luciliae* (Kallestad, MN, USA).

Results

Antiphospholipid reactivity

The ELISA detection of IgG aPL showed the presence of these antibodies in sera isolated from all five patients (1, 2, 3, 4 and 5) with quantitative values of 42, 30, 70, 64 and 25 GPL, respectively.

The ELISA detection of IgM aPL showed the presence of these antibodies in sera from patients 1, 3 and 5 with quantitative values of 32, 28 and 56 MPL, respectively.

The immunostaining for PL showed the presence of anti-CL antibodies (IgG) in patient 4 and anti-PS antibodies (IgG) in patient 3. These two sera had no IgG reactivity against all the other PL tested. Sera from patients 1, 2 and 5 showed no IgG binding to any PL (CL, PS, PE, PC, PI). IgM anti-CL were found in sera from patients 1, 3 and 5. No other IgM anti-PL antibodies were detected in these sera. Sera from blood donors did not show significant binding to all the PL tested (Fig. 1).

Anti-phospholipid binding protein antibodies

The ELISA for anti-PL binding protein antibodies (IgG) showed anti-prothrombin antibodies in patients 1 and 3, anti-annexin V antibodies in patient 3 and anti-β2-GPI antibodies in patient 4. None of the sera tested had significant binding to protein S. In parallel experiments, sera from patients with APS used as a positive control showed the expected reactivity (IgG) to β2-GPI, prothrombin and annexin V. Sera from blood donors did not show significant binding to any of the cofactor proteins tested (Fig. 2).

Antinuclear reactivity under standard condition and after apoptosis induction

The IIF previously carried out on Hep2 cells was then performed on fixed U937 cells under standard culture conditions with the 5 sera from the patients. On both cell substrates, the IIF

showed a coarse speckled antinuclear pattern. After apoptosis induction, IIF with these sera displayed either a higher intensity or the property of staining nuclear structures during fragmentation and redistribution towards cytoplasm and plasma membrane. Cytofluorimetric analysis showed DNA fragmentation occurring after apoptosis induction (Fig. 3). The binding properties of sera from patients (1-5) to antigens translocated to the plasma membrane was confirmed with an IIF on unfixed U937 cells followed by cytofluorimetric analysis (Fig. 4); controls showed no such binding.

Immunoreactivity against Hep2 total cell protein extract

The reactivity found in the sera of the 5 patients was characterized as being directed to a broad set of different antigens. Sera from patients 1-5 showed reactivity against bands within a wide range of MW(from 100 kD to 15 kD) and each serum displayed a unique pattern of reactivity. Serum from patient 4 dispayed only a few faint bands (Fig. 5). No binding was detected for the negative controls. Positive controls represented by sera from patients with autoimmune diseases showed a broad reactivity as expected.

Identified autoantigens reactivity

Sera from patients 1-5 showed no significant binding against the set of antigens tested (SmB, RNP-70k, RNP-A,

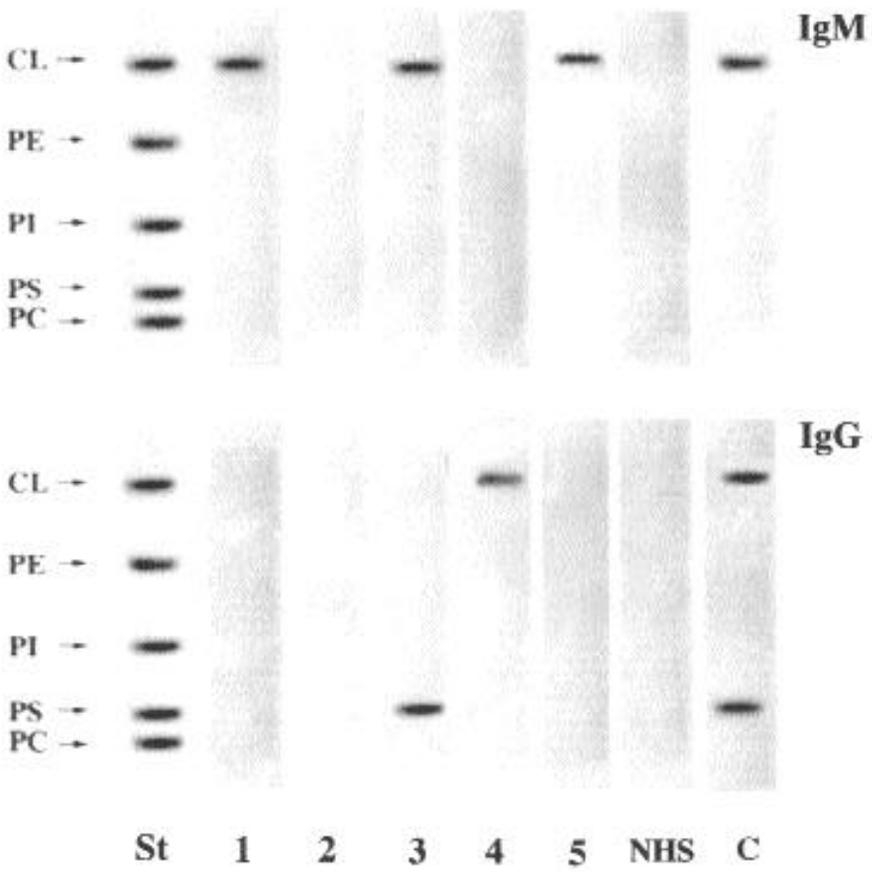

Fig. 1. Antiphospholipid antibodies by HPTLC immunostaining.
Immunostaining was performed using 2 µg of each phospholipid as antigen separated with HPLC aluminium-backed silica gel 60 (20x20) plates. Sera were diluted 1/100 in 0.5% gelatin/PBS. Horseradish peroxidase-conjugated goat anti-human IgG detector antibodies were diluted 1/500 in 0.5% gelatin/PBS. The colour reaction was developed by adding sodium nitroprusside.
St: Standard phospholipids separated by HPTLC. Lane 1-5: HPTLC and immunostaining with sera from patients 1,2,3,4,5. NHS:HPTLC and immunostaining with normal human serum. C:HPTLC and immunostaining with a serum from a patient with primary APS (positive control).
CL: cardiolipin; PS:phosphatidylserine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PC: phosphatidylcholine.

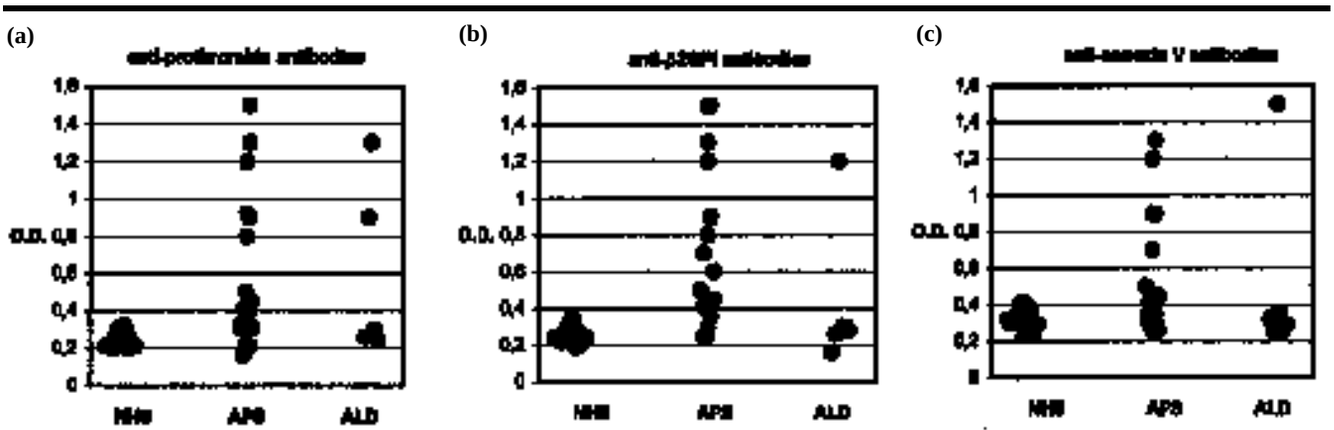

Fig. 2. Anti-phospholipid binding protein ELISA.
Anti-prothrombin (**a**),anti-β2GPI (**b**),and anti-annexin V (**c**) antibodies were detected in some sera from ALD patients with an ELISA method and compared with the reactivity of sera from patients with primary APS or blood donors (NHS). Anti-prothrombin antibodies were found in sera from patient 1 and 3,anti-β2GPI antibodies in serum from patient 4, and anti-annexin V in serum from patient 3.

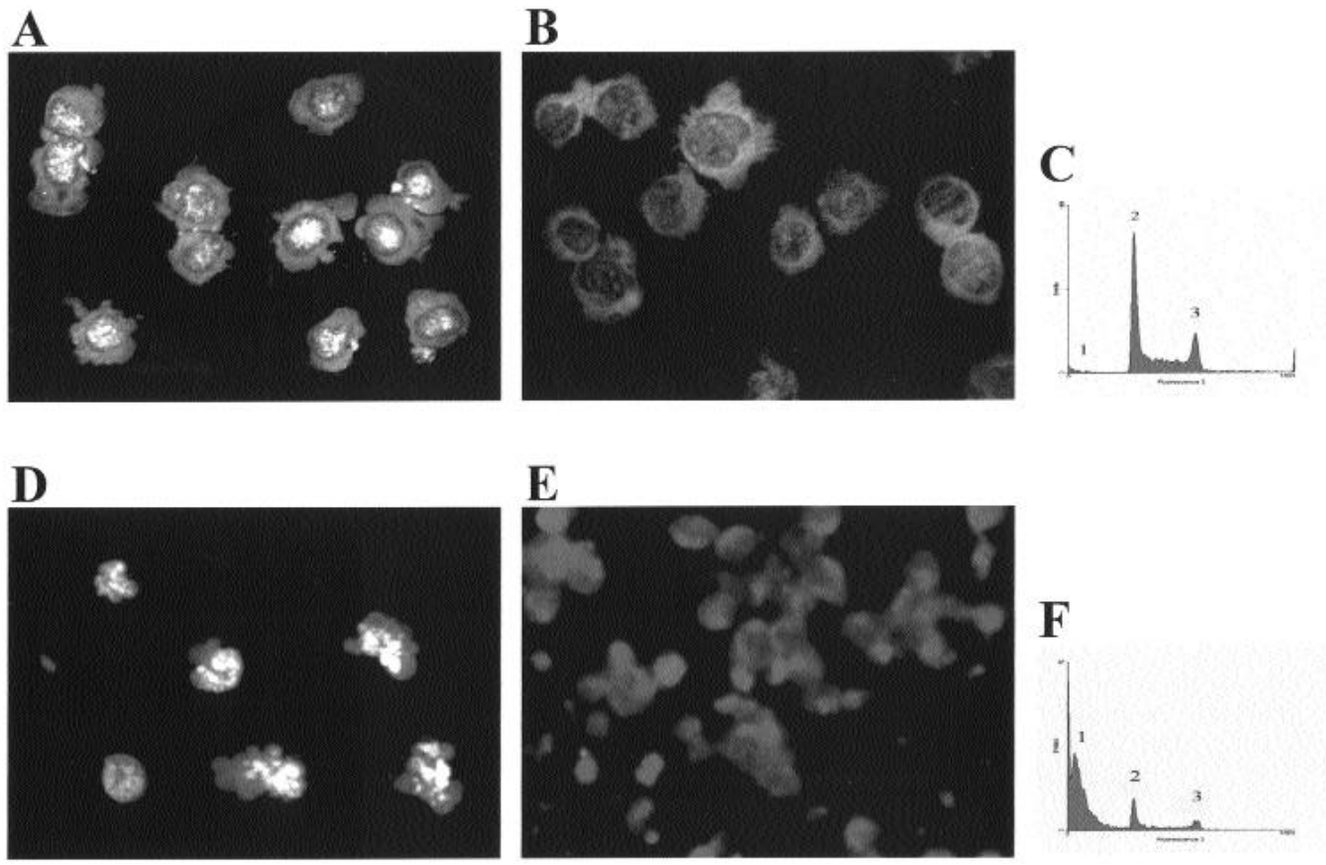

Fig. 3. Indirect immunofluorescence on apoptotic and viable U937 cells.
Sera from the patients studied (1-5) were tested with an IIF method on U937 cells under standard conditions and after the addition of the apoptotic stimulus. U937 cells were also stained with propidium iodide and analysed for DNA fragmentation by cytofluorimetric analysis.

A) The IIF performed on U937 cells under standard culture conditions with serum from patient 2 shows a coarse speckled nuclear pattern sparing cytoplasm and cell plasma membrane. A comparable pattern was observed with the other sera tested (1, 3, 4, 5).
B) The IIF performed on U937 cells under standard culture conditions with a normal human serum shows no significant fluorescence.
C) The cytofluorimetic analysis shows that under standard culture conditions while a negligible amount of cells display a hypodyploid DNA content (peak 1), the majority of cells have a dyploid (peak 2) or tetraploid (peak 3) DNA content. y-axis: cell number. x-axis: fluorescence intensity.
D) The IIF performed on apoptotic U937 with serum from patient 2 shows fragmentation and redistribution of fluorescence through cytoplasm and cell plasma membrane. A similar fluorescence was observed in the other sera tested (1, 3, 4, 5).
E) The IIF performed on apoptotic U937 with a normal human serum shows no significant fluorescence.
F) The cytofluorimetic analysis shows that after apoptosis induction, most of cells display a hypodyploid DNA content (peak 1), while a relatively reduced amount of cells with dyploid (peak 2) or tetraploid (peak 3) DNA content is observed. y-axis: cell number. x-axis: fluorescence intensity.

RNP-C, Ro52, SSB/La, CenpB, Topo-I, Jo1, SmD, ribosomal P protein, Ro60, Histons). Sera from patients with autoimmune diseases showed a binding which was in accordance with the expected pattern as the most commonly found in these diseases. The 8 sera from blood donors did not show significant binding (data not shown).

Discussion

It has been suggested that the abnormality of apoptosis may affect the establishment of correct tolerance towards self-antigens. Although some antinuclear antibodies are considered pathogenic, they could also be immunologic imprints of events linked to mechanisms of autoimmunity. It is widely accepted that each systemic autoimmune disease is associated with a characteristic autoantibody profile. Autoantibodies including aPL have been described in animal models with a structural defect of the Fas molecule and in their human counterparts, although in these patients the autoantibodies have not been fully characterized as yet (17). The defect in the Fas-signalling pathway, in the absence of any mutation of this molecule as it is found in ALD, drew our attention since this abnormality has recently been described in classic autoimmune diseases (10, 18, 19).

The elucidation of the molecular structure of nuclear antigens has revealed that most of them are components of subcellular particles and are often located in functional sites, as evidenced by the ability of autoantibodies to inhibit the function of these particles. These features have led to the concept that ANA are driven by subcellular components which are in an activated or functional state. It was reported that serum nucleosomal DNA naturally occurs in MRL *lpr/lpr* mice and that the appearance of this nucleosomal-DNA correlates with apoptosis in the thymus (20). Moreover, the accelerated apoptosis of circulating lymphocytes

sfrom patients with SLE was accompanied by the release of nucleosomes (21). Studies by Rosen and colleagues have indicated possible mechanisms by which subcellular particles are released from cells during apoptosis to become immunogenic (22).

Our study provides evidence that in the course of ALD a broad variety of ANA are produced and that the targets of these antibodies undergo a substantial change in their localization from the nucleus to the cytoplasm and plasma membrane, as suggested by the IIF experiments performed on either fixed and unfixed cells. Most of these antigens play a role in the apoptotic process or are redistributed during apoptosis from the nucleus to the cytoplasm and plasma membrane (23-25), which may provide an antigenic source to the immune system. However, although the sera from each patient showed a broad immunoreactivity against nuclear antigens, no clearcut recognition of the antigens commonly associated with connective tissue diseases such as ENA was detected. Furthermore, although DNA is one of the nuclear components commonly found on the surface of apoptotic cells, no anti-dsDNA antibodies were found in the sera of these patients. These findings suggest that other pathogenic factors, such as a specific cytokine *milieu* or the evolution of the autoimmune response through the steps of epitope spreading, may be needed for the generation of these specific immune responses.

This study represents the first characterization of the aPL found in ALD. The combined use of HPTLC immunostaining, which permits a highly accurate definition of PL specificity in the absence of protein contamination and of ELISA with delipidated cofactor proteins, allows a fine analysis of aPL specificity. This analysis provides evidence that aPL found in ALD include antibodies recognizing "pure" PL molecules and antibodies directed against cofactor proteins such as β2-GPI, annexin V and prothrombin in analogy with what was found in sera from patients with APS (14, 26). Interestingly, the autoantibodies found in these patients selectively target negatively-charged (CL and PS) but not neutral PL (PE and PC). In addition, in serum from patient 2 aPL were detectable with a traditional aPL ELISA, but not with a HPTLC-immunostaining or with an anti-phospholipid binding protein ELISA, a finding which confirms the previously reported differences between the three methods (14).

It is now widely accepted that the sudden loss of the normal PL distribution in plasma membrane and the consequent exposure of PS, a negatively-charged PL, is a specific and early sign of cell commitment to apoptosis (27). Furthermore, our recent study reinforces the concept that CL may be significantly involved in the induction of aPL by apoptotic cells since this molecule, normally confined to the inner mitochondrial membrane, is exposed on the plasma membrane in the early phases of the apoptotic process (28). It is of interest to observe that these patients produce antibodies against the cofactor proteins which play potentially relevant (β2-GPI and annexin V) or already known (prothrombin) functions

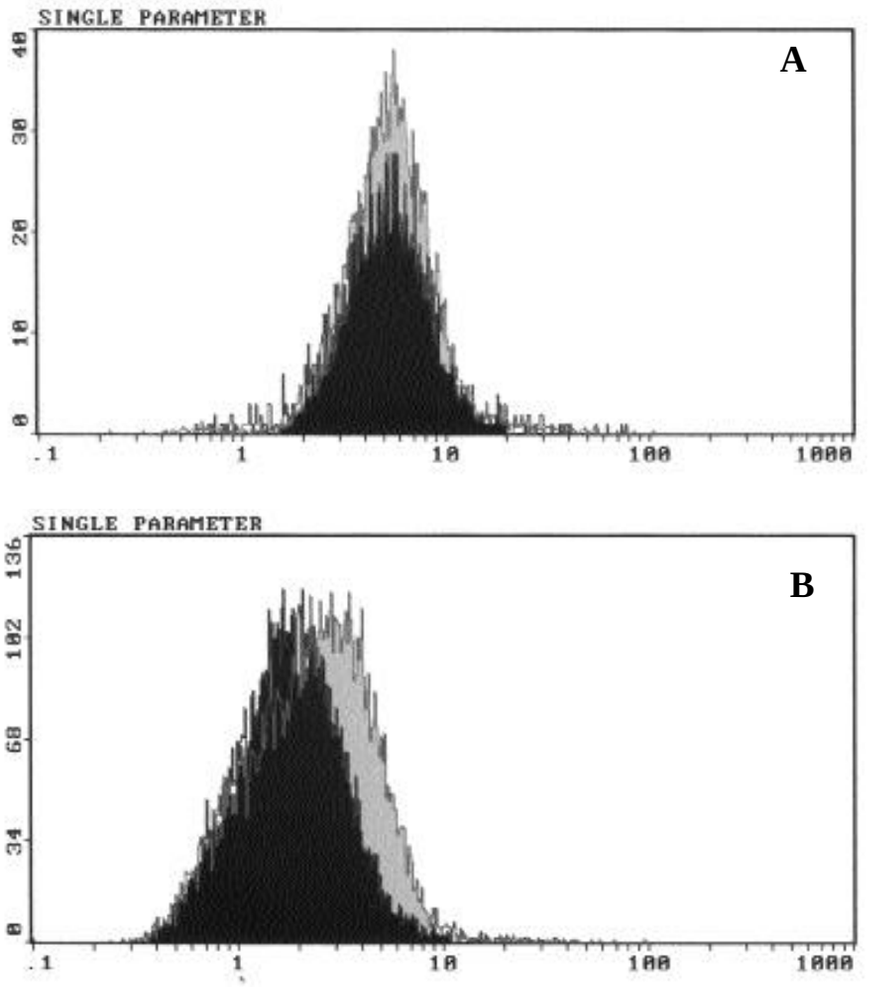

Fig. 4. Indirect immunofluorescence and cytofluorimetic analysis on unfixed apoptotic and viable U937 cells.

An IIF with sera from patients and contols on unfixed U937 cells followed by a cytofluorimetric analysis was carried out to verify the capacity to bind to plasma membrane structures in the course of the apoptotic process. The cytofluorimetric analysis of the IIF obtained with the serum from patient 3 as against that obtained with a normal human serum is shown. Comparable results were obtained with the analysis of the sera from the other patients (1,2,4,5). Black histogram:binding to viable cells. Grey histogram: binding to apoptotic cells.

(**A**) The binding of a normal human serum (NHS) to viable and apoptotic cells does not significantly differ.

(**B**) Cell binding of serum from patient 3 increases after apoptosis induction.

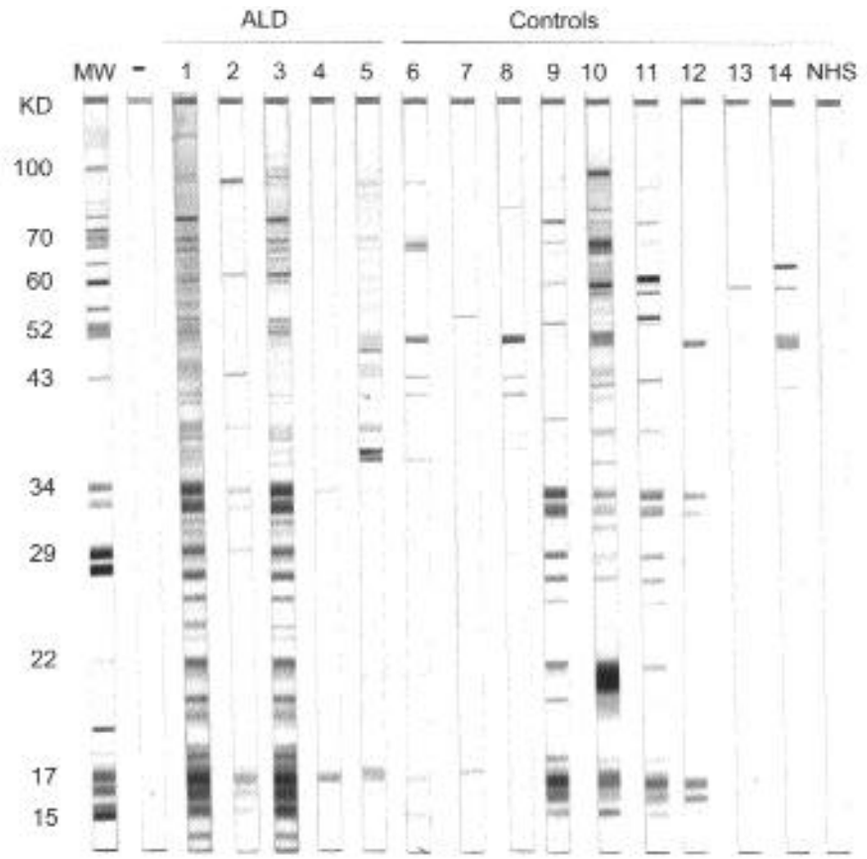

Fig. 5. Immunoreactivity against Hep2 total cell protein extract.
Sera from patients 1-5 with ALD and a set of sera from patients with autoimmune diseases (6-14) including SLE (6,9,12,13), PSS (8) overlap of SLE and PSS (10), PM (7), DM (11), SS (14) and a serum from a blood donor (NHS) were tested on an Hep2 total protein cell extract Western Blot.
MW: molecular weight markers. (-): negative control enclosed in the test

in coagulation homeostasis.
It has been suggested that the subsets of aPL which are actually directed against β2-GPI (29), prothrombin (30) and possibly annexin V (31) are more closely related to the clinical features of APS than classic aPL taken as a whole, although autoantibodies targeting PL molecules have also been found relatively often in autoimmune conditions (14). Furthermore, evidence was provided that both annexin V and β2-GPI bind to negatively-charged PL exposed in the early phases of apoptosis and thereby participate to the clustering of autoantigens found in the apoptotic bodies (32,33). The ability of prothrombin to bind to PL in the context of a procoagulant "milieu" makes it very likely that this protein also displays the ability to bind to apoptotic cells. The absence of anti-protein S antibodies parallels what seen in sera from patients with APS in which these antibodies are rarely found.
Despite the heterogeneity of the aPL response observed in the sera of these patients, the detection of aPL was not a transient phenomenon as observed in the course of acute infections but was found in assays conducted 2 weeks apart, as it is generally suggested to confirm this positivity, and was continuously detected later over a period of years. In addition, plasma isolated from all patients displayed prolonged aPTT, confirmed over time, which may suggest an impact of these autoantibodies on coagulation parameters (unpublished observation).
Although this human disorder seems to be associated with the appearance of a wide spectrum of different autoantibodies, some of them occurring in the same patient, no clinical features were associated with these specificities. Furthermore, despite the continous detection of aPL in the sera of these patients, no clinical manifestations of APS (arterial/venous thrombosis) were reported.
Our observation is in keeping with the hypothesis that an impairment of Fas-mediated apoptosis is involved in the generation of autoimmune phenomena such as the production of a variety of autoantibodies, but this event is not *per se* sufficient to the development of an overt connective tissue disease or antiphospholipid syndrome with its distinctive clinical features. The thrombocytopenia reported in the clinical history of the 5 patients, although often associated with the detection of aPL, cannot be directly attributed to the presence of these autoantibodies. Other concomitant clinical events associated with the lymphoproliferative disease or other autoantibodies may significantly or exclusively contribute to this condition. In this respect, the autoimmune phenomena observed in these patients were mainly haematologic and a recent study highlighted the presence of autoantibodies against hematopoietic cell lineages in the course of ALPS (34).
In conclusion, this study shows that in the presence of the functional defect of Fas-induced apoptosis, autoantibodies against a broad set of autoantigens potentially involved in apoptosis are produced. The question as to whether this defect is involved in either the pathogenesis of autoimmune diseases or in the general mechanism of autoantibody production awaits further investigation.

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