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# Autoantibodies to Nuclear Antigens

*Correlation Between Cytotoxicity and DNA-Hydrolyzing Activity*

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## Abstract

The cytotoxicity of DNA-specific autoantibodies from sera of patients with systemic lupus erythematosis (SLE) and with lymphoproliferative diseases, and from blood of healthy donors was examined on tumor-cell lines L929 and HL-60. DNA-binding IgG fractions from SLE and chronic lymphocytic leukemia (CLL) sera were cytotoxic at concentrations of up to $10^{-10}$ *M*. No detectable changes in cell viability were observed after incubation with antibodies devoid of DNA-binding activity and DNA-specific antibodies isolated from blood of healthy donors and patients with T-cell lymphoma, B-cell lymphosarcoma, and acute B-cell leukemia. There was good correlation between the cytotoxic activity and DNA-hydrolyzing activity of anti-DNA antibodies. The cytotoxic effect of DNA-binding antibodies presumably was complement-independent, because it was attributed only to the Fab fragment. The cytotoxic effect was completely inhibited by preincubation with double-stranded DNA (dsDNA). Both the cytotoxic effect and the DNA-hydrolyzing activity of anti-DNA antibodies were significantly increased in the antibody fraction that displayed cross-reactivity with nuclear matrix proteins. Possible mechanisms for the formation and pathogenicity of cytotoxic anti-DNA antibodies are discussed in this article.

**Index Entries:** Anti-DNA antibodies; catalytic autoantibodies; cytotoxicity; nuclear antigens.

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## Introduction

Research carried out during the last decade challenges the concept that antigen binding is the sole function of antibodies. Investigations of antibody catalytic properties *(1)*—together with the data on the formation of antibodies to vitally important cellular membrane antigens, such as Fas and TNF-α receptors *(2,3)*—suggest that antibodies can share the structural peculiarities and mimic the actions of both enzymes and regulatory molecules. The activity of these antibodies or superantibodies *(4)* could contribute to the fundamental mechanisms of healthy immunity and pathological alterations in the course of autoimmune disease.

The discovery of the ability of antibodies to penetrate the living cell and the nucleus *(5)* allows one to hypothesize the involvement of antibodies in intracellular processes. Study of the specificity of antibodies crossing the cell membrane should provide clues for understanding of the role of these antibodies inside the cell. Most experimental data indicate that among the different antibody species, anti-DNA and anti-RNP antibodies most frequently bear the cell-penetrating capacity *(6,7)*.

Anti-DNA antibodies are usually present at low levels in blood of healthy individuals, and their quantity often increases in patients with autoimmune disorders *(8)*. A number of anti-DNA antibodies have been described that cross-react with other nuclear antigens such as histones and nuclear-matrix proteins *(9,10)* and with membrane antigens *(11)*. Recent data indicate that an increased polyspecificity of anti-DNA antibodies correlates with their ability to enter the cell *(12)*.

Certain anti-DNA autoantibodies from sera of patients with autoimmune pathologies and chronic lymphoproliferative diseases have revealed catalytic activity toward double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) *(13)*. However, the pathogenic significance of this antibody property remains elusive. Previously, we have reported the cytotoxic effect of anti-DNA antibodies from sera of patients with autoimmune diseases, and an increased level of DNA-hydrolyzing activity in DNA-binding IgG fraction cross-reactive with nuclear-matrix proteins *(14)*. Here we hypothesize that autoimmune disorders may promote formation of antibodies that, reacting with nuclear antigens, display DNA-hydrolyzing properties and exert cytotoxic effects. In this article, we discuss possible mechanisms of the formation and pathogenicity of these antibodies in autoimmune disease.

## Materials and Methods

### *Materials*

Blood samples of patients with lymphoproliferative and autoimmune diseases and those of healthy donors were provided by the physicians of the Russian Haematological Center, Institute of Rheumatology, and Moscow Regional Research Institute of Clinical Investigations.

Salts and chemicals were from Sigma and Merck. Chromatography equipment and sorbents were from Pharmacia. Cell-culture materials were from Life Technologies, Inc. Agonistic anti-Fas receptor antibody IPO-4 was the gift of Prof. S.P. Sidorenko and Dr. S.V. Mikhalap (Kavetsky Institute of Pathology, Oncology, and Radiobiology, Kiev, Ukraine).

### *Purification of DNA-Binding IgG and Their Subfragments and Analysis of DNA-Hydrolyzing Properties*

Antibodies were precipitated from serum fluid with ammonium sulfate (40%), and further steps of purification were conducted essentially as described in *(15,16)*. The homogeneity of the FPLC-grade final preparation was checked by silver-stained SDS-PAGE as described by Laemmli *(17)* and immunoblotting with mouse antihuman IgG antibodies (Sigma) (data not shown). DNA-hydrolyzing activity was assayed by monitoring conversion of supercoiled plasmid DNA into circular or linear form as previously described *(13,16)*. Supercoiled plasmid DNA (pUC19) was isolated as described in *(18)*. Full conversion of 1 μg of supercoiled plasmid DNA into circular form after 10 h of incubation with antibodies in buffer containing 20 m*M* Tris-HCl, 50 m*M* NaCl, and 10 m*M* $MgCl_2$ was considered as 1 U of DNA-hydrolyzing activity.

In order to exclude the possibility of any contamination of the antibody samples, the preparations of anti-DNA antibodies were subjected to gel-filtration on Superdex 75 column (Pharmacia) in 1 *M* acetic acid, 100 m*M* NaCl ("acid shock"). The pH of the effluent antibody fraction was brought to 7.5 by 1 *M* Tris-base.

Fab and Fc fragments of polyclonal IgG preparation were obtained by papain digestion as described in *(19)* and separated by chromatography on Pharmacia HR 5/5 MonoQ column in buffer, containing 10 m*M* Tris-HCl, pH 8.8, and 25 m*M* NaCl. Under these conditions, Fab fragments eluted in the column flow-through, and the Fc fragments were eluted from the column by an NaCl gradient (25 m*M*–1 *M*). Both fractions were further transferred into a phosphate-buffered saline solution (PBS). Separation from noncleaved IgG was done by gel-filtration on the Superdex 75 column (Pharmacia). Purity of final Fab and Fc fragment preparations was confirmed by silver-stained SDS-PAGE.

### *Cytotoxicity Assays and Inhibition of Antibody Cytotoxicity by DNA*

Tumor-cell lines HL-60 (human promyelocytes) and L-929 (mouse fibroblasts) were obtained from ATCC and cultured in RPMI 1640 media containing 2 m*M* L-glutamine, 10 mg/mL gentamicin, and 10% heat-inactivated fetal bovine serum. The amount of dead cells in culture did not exceed 5%. Purified antibody preparations were dialyzed twice against 2000 vol of PBS before addition to the cells.

Thirty thousand target cells in 100 μL of RPMI 1640 were incubated with antibodies at concentrations ranging from $10^{-12}$ to $10^{-6}$ *M* at one order

of magnitude increments. Each dilution was repeated eight times in separate wells. Each experiment was repeated five times, and the average deviation in the percentage of dead cells was determined between separate wells and between the repeats. The resulting average deviation did not exceed 3% from the input cell number.

Mock reactions were performed in 100 μL of RPMI 1640 medium in the absence of antibodies. Incubation time varied from 3 to 48 h, depending on the purpose of the experiment. Trypan-blue inclusion (as described in *[20]*) and 3(4,5–dimethylthiazol–2yl)2,5–diphenyltetrazoliumbromide (MTT) assays were used to calculate the percentage of dead cells after incubation. To perform MTT assay (colorimetric cell-viability test based on conversion of tetrazolium salts to formazan crystals by mitochondrial enzymes), cells cultured in the presence of antibodies were incubated with the MTT reagent (Boehringer Mannheim, Mannheim, Germany) before extraction with DMSO. Absorbance was recorded at 590 nm using ELISA reader (Labsystems). Absorbance values were normalized by subtracting the background in the absence of cells. The quantity of dead cells was calculated as the percentage of control cells cultured in the absence of antibodies.

To inhibit the cytotoxic activity, $10^{-7}$ *M* of FPLC-grade anti-DNA antibodies from patients with chronic lymphocytic leukemia were preincubated with 1 μg of supercoiled plasmid pUC19. Incubation was conducted in 20 μL of PBS for 1 h at room temperature with shaking. Cytotoxicity of antibodies preincubated with DNA was compared with that of the initial antibody preparation taken at the same concentration and adjusted to the volume of 20 μL with sterile PBS. Mock reactions were performed with 20 μL of PBS alone and 20 μL of PBS containing 1 μg of plasmid. No cytotoxicity was displayed by dsDNA alone.

### *Preparation of Nuclear Matrix Affinity Support and Affinity Chromatography on Nuclear Matrix Column*

Nuclear-matrix proteins (NMPs) were isolated as described in *(21)*. Preparation of the affinity support harboring the proteins and removal of residual genomic DNA was performed as reported previously *(20)*. DNA-free NMP-bearing support was used in further experiments.

Purified IgG from autoimmune sera were applied to the nuclear matrix protein-Sepharose (NMP-Sepharose) HR 5/2 FPLC column in 20 m*M* Tris-HCl, pH 7.6, 50 m*M* NaCl. The matrix-bound fraction was eluted with 100 m*M* glycine-HCl, pH 2.6, followed by neutralization of the effluent with 1 *M* Tris-HCl, pH 9. The nuclear-matrix-bound antibody fractions and unbound fractions were dialyzed against 2000 vol of PBS overnight and tested for cytotoxic activity.

### *Analysis of Cross-Reactivity of anti-DNA Antibodies with Membrane and Nuclear Matrix Proteins*

To obtain membrane proteins, 5 × 109 HL-60 cells were collected and washed three times with PBS by centrifugation at 2000 rpm. Further pro-

cedures were conducted on ice. The cells were suspended in ice-cold lysis buffer (10 m*M* KCl, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl pH 7.4, and 2 m*M* phenylmethysulfonyl fluoride [PMSF]). Cells were broken in Dounce homogenizer by 15 strokes of a "tight" pestle. The completion of the procedure was judged under the microscope. The lysate was centrifuged at 7800 rpm for 10 min to pellet the nuclei. The supernatant was collected and centrifuged at 38000 rpm for 1 h at 0°C. The pellet (heavy membrane fraction mostly containing cellular organelles) was resuspended in 0.5 mL of lysis buffer and analyzed for protein content by SDS-PAGE. The remaining supernatant (enriched in plasma membrane proteins) was divided into 100 μL aliquots and lyophilized overnight.

Nuclear-matrix and membrane proteins (10 μg of each per lane) were resolved by SDS-PAGE. Immunoblotting was conducted for 1 h in Transblotter device (Pharmacia) according to the standard technique *(22)* with current stabilized at 1 mA per 1 $cm^2$ of the gel. The transfer membrane (Hybond C-Extra, Amersham) was incubated for 2 h with blocking buffer containing 5% bovine serum albumin (Sigma), 0.7% Tween 20, 0.15 *M* NaCl, 20 m*M* Tris-HCl pH 7.5. Antibodies were added to the membrane at concentration 0.5 μg per mL in 0.15 *M* NaCl, 20 m*M* Tris-HCl, pH 7.5, 0.5% of bovine serum albumin (Sigma). After 6 h of incubation with antibodies and three subsequent washes with buffer containing 0.7% Tween 20, 0.15 *M* NaCl, 20 m*M* Tris-HCl, pH 7.5, the membrane was stained with mouse antihuman IgG (Fc-fragment specific) peroxidase-labeled antibodies (Sigma) in accordance with recommendations of the manufacturer and developed using HRP Conjugate Substrate Kit (Bio-Rad). No nonspecific binding of the second antibody was observed to nuclear-matrix and membrane proteins judging in the control assays.

## Results and Discussion

### *Cytotoxicity of Anti-DNA Autoantibodies from Sera of Patients with Systemic Lupus Erythematosus and Lymphoproliferative Diseases*

The pathogenic role of anti-DNA autoantibodies in autoimmune diseases is described in a number of studies *(23–25)*. Recent data indicate that along with the formation of the immune complexes involved in the development of lupus nephritis *(26)*, anti-DNA antibodies appear to be cytotoxic to primary cultures of lymphocytes *(27)*, and may also cause apoptosis in cultured mesangial cells *(28)*. In some cases, anti-DNA antibody-mediated tissue injury is proposed to occur because of the penetration of the antibodies into living cells *(29)*.

We investigated the possible physiological role of the DNA-cleaving catalysts. We tested the cytotoxicity of DNA-specific antibodies isolated from sera of 16 patients with systemic lupus erythematosus (SLE), 10 patients with chronic lymphocytic leukemia (CLL), 12 patients with acute B-cell leukemia, 15 patients with various types of T-cell proliferation (T-cell lymphoma and acute T-cell leukemia), 12 patients with B-cell lym-

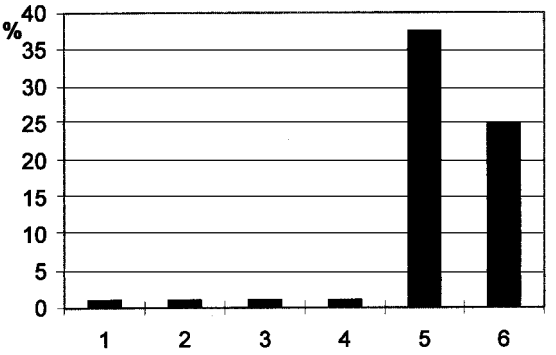


Fig. 1. The occurrence of cytotoxic anti-DNA antibodies in sera of patients with SLE, lymphoproliferative diseases, and healthy donors. Antibody cytotoxicity was assayed using tumor-cell line L929 (mouse fibroblasts). Percentage of dead cells was calculated based on trypan-blue exclusion by cells after 48 h incubation with $10^{-7}$ *M* anti-DNA antibodies. The vertical axis shows the percent patients displaying anti-DNA antibody cytotoxicity. 1: Healthy donors; 2: acute B-cell leukemia; 3: B-cell lymphosarcoma; 4: T-cell proliferation; 5: systemic lupus erythematosus; and 6: chronic lymphocytic leukemia.

phosarcoma, and 10 healthy donors. Figure 1 presents the results of primary screening for cytotoxicity of anti-DNA antibodies obtained using L929 cells and the trypan-blue exclusion assay. Anti-DNA antibodies from patients with acute B-cell leukemia, B-cell lymphosarcoma, and T-cell proliferation and from healthy donors did not display cytotoxicity. Similarly, no cytotoxic effect was displayed by the same amount of antibodies devoid of DNA-binding activity. On the contrary, cell mortality ranging from 15% to 35% was observed after incubation with anti-DNA antibodies from six patients with SLE and three patients with CLL. Interestingly, all three patients with CLL had concomitant autoimmune syndromes (autoimmune hemolytic anemia, thrombocytopenia, dermatitis). Thus, one can suggest that the production of cytotoxic anti-DNA antibodies is linked to autoimmune disorders.

Our data indicate that certain DNA-binding antibodies from SLE and CLL sera were cytotoxic at concentrations of up to $10^{-10}$ *M (20)*. Usually, however, the cytotoxic effects were observed at higher antibody concentrations ($10^{-8}$–$10^{-7}$ *M*). These antibody concentrations were employed in further experiments.

To exclude possible cytotoxic effects of serum factors potentially associated with autoantibodies, we compared the cytotoxicity mediated by acid-shocked antibody samples and of the initial anti-DNA antibody preparations at equal protein concentration ($10^{-8}$ *M*) on tumor cells L929 and HL-60 (human promyelocytes) using the MTT assay (*see* Fig. 2). No difference in the level of cytotoxic effect was observed between the "acid-

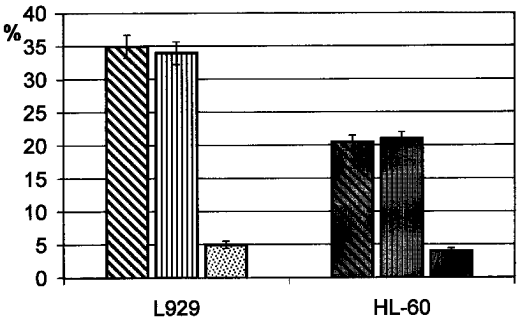


Fig. 2. Cytotoxic effect of anti-DNA antibodies from SLE patient on L929 and HL-60 tumor cells. Vertical axis shows the percent dead cells after 48 h incubation with $10^{-8}$ *M* antibodies (angled lined bars), $10^{-8}$ *M* of "acid-shock"-treated antibodies (vertically lined bars), with medium alone (dotted bars).

shock"-treated samples and the initial anti-DNA antibody preparations.

The cytotoxic effect of the anti-DNA antibodies was potent on L929 cells (an epithelial cell line) compared to HL-60 cells (a macrophage cell line) (35% and 21% of dead cells, respectively). This is not surprising, because skin and epithelial lesions in autoimmune pathologies are widespread *(30)*, while direct macrophage damage has never been detected in autoimmune disease.

A critical point for investigation of the mechanism of antibody cytotoxicity is to determine the role of activation of complement. Physiological interaction of antibodies with the living cell is frequently mediated by the Fc fragment *(31)*. Moreover, binding of the Fc fragment to the C1q component of the C1 complex is the crucial step for activation of complement system by classical pathway *(32)*.

We compared the cytotoxic effects of Fab and Fc fragments of antibodies isolated from sera of the patients selected by primary screening of anti-DNA antibody cytotoxicity. The DNA-binding and non-DNA binding Fab fragments were separated using chromatography on DNA-Sephacryl. DNA-binding Fab fragments, Fc fragments, and DNA nonbinding Fab fragments in equimolar concentration ($10^{-7}$ *M*) were assayed for cytotoxicity using HL-60 cell line in the absence of complement (*see* Fig. 3). Incubation of cells with DNA-binding Fab fragment resulted in significant cell mortality (23% of dead cells). In contrast, no decrease in cell viability was induced by incubation with DNA nonbinding Fab fragments or Fc fragments. Thus, cytotoxicity exerted by DNA-binding antibodies is attributed exclusively to Fab fragments, suggesting the complement-independent nature of the effect.

The direct link between specificity for DNA and the cytotoxic activity displayed by autoantibodies was studied. Since both cytotoxic activity

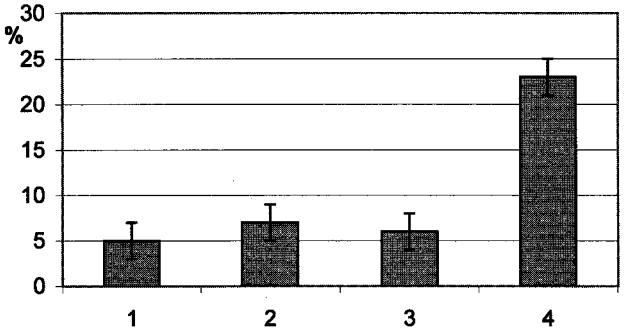


Fig. 3. Cytotoxicity of DNA-binding antibody Fab fragments. Vertical axis shows the percentage of dead cells after 48 h incubation. Antibodies were isolated from serum of CLL patient and separated into Fab and Fc fragments as described in Materials and Methods section. HL-60 cells were incubated with media alone (1), with $10^{-7}$ *M* of DNA nonbinding antibody Fab fragments (2), $10^{-7}$ *M* of antibody Fc fragments (3), and $10^{-7}$ *M* of DNA-binding antibody Fab fragments (4).

and DNA-binding activity of the anti-DNA antibodies were localized to their Fab fragments, we expected that the cytotoxic effect should be inhibited by preincubation with DNA. Cytotoxicity by antibodies preincubated with DNA and the same amount of initial anti-DNA antibodies was compared (*see* Fig. 4). Complete inhibition of the cytotoxic effect by preincubation of antibodies with DNA was evident. This result is in full agreement with the data from primary cultures of lymphocytes treated with monoclonal anti-DNA antibodies *(27)*. In the same vein are previous results showing that loss of antibody DNA-binding capacity after site-directed mutagenesis blocked the ability of an antibody to penetrate the membrane of a living cell *(33)*.

Our experiments indicate that anti-DNA autoantibodies produced in systemic autoimmune disorders can be cytotoxic for cultured cell lines. At least two possible mechanisms could be suggested to explain the phenomenon of anti-DNA antibody-mediated cytotoxicity. First, a high level of polyspecificity of anti-DNA antibodies presumably permits their interaction with targets on the cell membrane, possibly inducing signal transduction events which lead to cellular death. The membrane target molecule in this case might be an as-yet undetermined target or the well-described cell death receptors, such as Fas, and TNF-α. Data on the presence of natural anti-Fas antibodies in intravenous IgG preparations (IVIg) derived from healthy donors *(2)* favors this hypothesis. Alternatively, cell death could be mediated by antibody penetration into living cells. Cross-reactive anti-dsDNA antibodies entering the cytoplasm could potentially serve as inhibitors of protein synthesis *(34)*. In case of antibody entry into the nucleus, the DNA-hydrolyzing properties of antibodies could be fatal for cells.

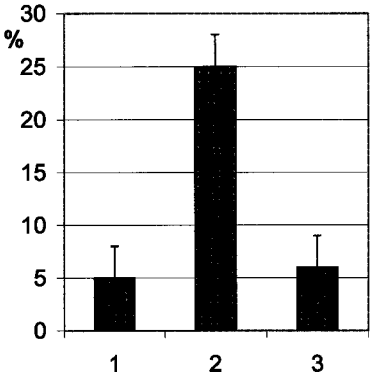


Fig. 4. Inhibition of cytotoxic activity of DNA-binding antibodies by dsDNA. Vertical axis shows the percentage of dead cells. HL-60 cells were incubated for 48 h with medium alone (1), $10^{-7}$ *M* DNA-binding antibodies from SLE patient (2), $10^{-7}$ *M* DNA-binding antibodies from SLE patient preincubated for 1 h with 1μg of supercoiled plasmid DNA (3).

## *Cytotoxic and DNA-Hydrolyzing anti-DNA Antibodies Cross-React with Nuclear-Matrix and Membrane Antigens*

Cross-reactions with other self-antigens is a characteristic feature of autoantibodies *(35)*. Anti-DNA antibodies frequently bind histones *(9)*, cytoskeleton components *(36)*, and phospholipids *(37)*. Moreover, the formation of anti-DNA autoantibodies is thought to be induced by DNA-protein complexes containing a distorted high-energy ground state of the DNA molecule present in blood as a result of cellular destruction *(38)*. Thus, complexes of DNA and NMP may be major agents provoking formation of anti-DNA autoantibodies *(14)*. This notion has been supported by our previous studies describing the cross-reactivity of DNA-hydrolyzing antibodies with a 35-kDa nuclear-matrix protein *(14)* and the capture of DNA-hydrolyzing antibodies by an affinity column expressing immobilized NMP *(19)*. Encouraged by these data, we investigated the cytotoxic effects of antibodies captured by the NMP affinity support.

The cytotoxicity of nuclear-matrix-bound antibodies and unbound antibodies was assayed in equal concentrations ($10^{-8}$ *M*) using HL-60 cells. Cell viability was checked by the MTT test. The results of HL-60 cell treatment with NMP-specific autoantibodies and the column flow-through are presented in Fig. 5. Selective cytotoxic effects of the NMP-Sepharose binding antibodies was evident. Incubation of cells with NMP binding antibodies yielded 25% dead cells after 48 h of incubation, comparable to the cytotoxic effect of the initial anti-DNA antibodies from this patient applied at the same concentration (20% of dead cells). The cytotoxicity of NMP nonbinding and DNA nonbinding fractions was insignificant.

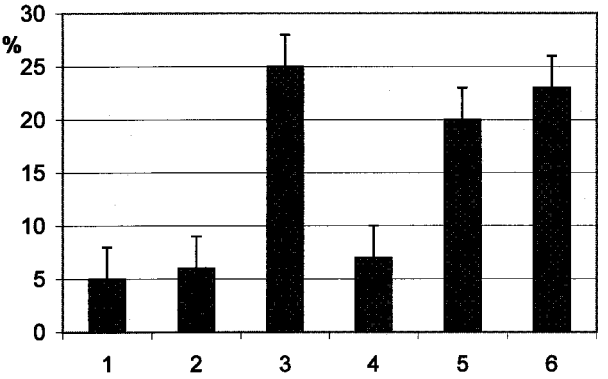


Fig. 5. Cytotoxicity of nuclear matrix protein-binding antibodies. Vertical axis shows percent dead cells. HL-60 cells were incubated for 48 h with medium alone (1), $10^{-8}$ *M* DNA nonbinding antibodies from an SLE patient (2), $10^{-8}$ *M* DNA-binding antibodies from an SLE patient (3), $10^{-8}$ *M* nuclear matrix protein non-binding antibodies from an SLE patient (4), $10^{-8}$ *M* of nuclear matrix protein binding antibodies from an SLE patient (5), and $10^{-7}$ *M* anti-Fas receptor antibody IPO-4 (6).

An antibody known to induce apoptosis (IPO-4) via interaction with Fas receptor *(39)* was employed as a positive control in cytotoxicity assays. As shown in Fig. 5, 48 h of incubation of cells with $10^{-8}$ *M* of IPO-4 resulted in 23% of dead cells. The fact that the cytotoxic effects of polyclonal SLE-derived anti-DNA and anti-NMP antibodies on a promyelocyte cell line are of the same order as the monoclonal cytotoxic antibody to human Fas receptor suggests that the former antibodies may contribute in the development of lymphocytopenia often observed in autoimmune diseases *(27)*.

Most case examples of external induction of cellular processes (including apoptosis) demand binding of ligand to cell-surface receptors. The ability of anti-DNA antibodies to induce cell death could indicate the existence of a surface receptor-like target for these antibodies. Anti-DNA antibodies are known to cross-react with a number of cell-surface antigens *(11)*. Binding of cross-reactive anti-DNA autoantibodies to receptors may trigger signal transduction mechanisms resulting in cell death. Moreover, the cytotoxicity of anti-DNA antibodies, along with the data suggesting apoptosis-inducing autoantibodies to Fas receptor in IVIg preparations *(2)* suggests involvement of naturally forming antibodies in regulation of vital cell functions.

We employed immunoblotting procedures to analyze the cross-reactivity of cytotoxic anti-DNA antibodies with membrane and nuclear-matrix proteins. Immunoblotting of membrane proteins resolved by SDS-PAGE and stained with cytotoxic anti-DNA antibodies revealed several bands with molecular weight ranging from 30 to 90 kDa (*see* Fig. 6). Interestingly, staining of NMPs by cytotoxic anti-DNA autoantibodies revealed two intense bands with approximate molecular weight of 30–35 kDa, con-

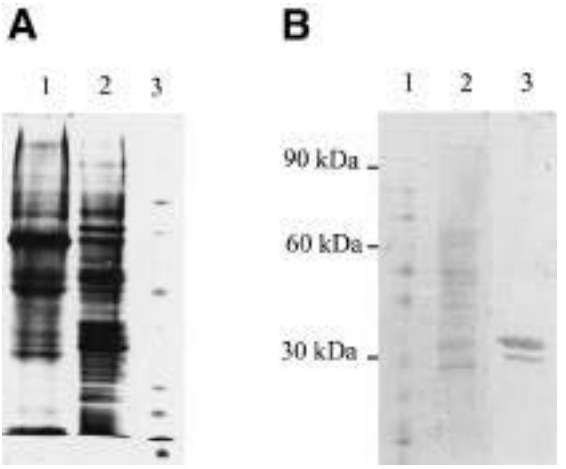


Fig. 6. Cross-reactivity of cytotoxic anti-DNA antibodies with nuclear-matrix and membrane proteins. **(A)** Coomassie-stained SDS-PAGE of membrane (lane 1) and nuclear matrix (lane 2) proteins (5 μg/lane). Lane 3, molecular weight markers (LKB). **(B)** Immunoblot of membrane (lane 2) and nuclear matrix (lane 3) proteins, staining with cytotoxic anti-DNA antibodies from serum of SLE patient (1 μg/mL of binding solution). Second antibody—peroxidase-labeled mouse antihuman Fc-fragment antibody (Sigma), peroxidase color reagent β-chloronapthol. Lane 1, prestained molecular weight markers (Life Technologies).

sistent with data on the cross-reactivity of DNA-hydrolyzing antibodies with nuclear-matrix antigens *(14)*.

Unfortunately, the polyclonal nature of cytotoxic anti-DNA antibody preparations and the destruction of conformational epitopes under denaturating electrophoresis conditions impose difficulties in determining the specific targets of these antibodies. Further experiments on native NMPs interacting with monoclonal cytotoxic anti-DNA antibodies may help to clarify the mechanisms of their penetration and effects.

### *Cytotoxicity of Anti-DNA Antibodies Correlates with DNA-Hydrolyzing Activity*

The spontaneous occurrence of catalytic antibodies is linked to autoimmune abnormalities *(13,40,41)*. However, the contribution of catalytic antibodies to the autoimmune pathological process remains to be determined. Presumably, proteolytic autoantibodies could contribute to the tissue damage by cleavage of important molecules involved in cellular interactions or exposed to the cell surface, e.g., proteinaceous messengers like cytokines. Natural DNA-hydrolyzing antibodies *per se* may not be pathogenic, because it is highly unlikely that cleavage of serum DNA severely impairs cellular functions. On the contrary, hydrolysis of nuclear DNA will be inevitably harmful to the cell. Thus, localization of DNA-hydrolyzing

antibodies to the nucleus is the most plausible pathway by which these antibodies may be pathogenic. Penetration of DNA-cleaving antibodies into the cell, and their concentration in the nucleus, could provoke DNA cleavage and result in cellular death. This seems possible, since a significant body of evidence regarding penetration of DNA-specific antibodies into living cells and subsequent nuclear localization has accumulated in recent years *(4–6)*.

We therefore investigated the DNA-hydrolyzing activity of cytotoxic anti-DNA antibodies from sera of SLE and CLL patients. Of six SLE patients with anti-DNA antibody cytotoxicity, five displayed DNA-hydrolyzing activity. All three CLL patients exerting anti-DNA antibody cytotoxicity were characterized by the presence of DNA-hydrolyzing antibodies. In comparison, screening for antibody DNA-hydrolyzing activity in patients without cytotoxic activity of antibodies revealed only one SLE patient and one CLL patient displaying catalytic activity. Thus, in most cases, anti-DNA antibody cytotoxicity was associated with the DNA-hydrolyzing activity. Further analysis of the cytotoxic effects and cell-penetrating capacity of monoclonal Fab fragments of DNA-hydrolyzing antibodies would provide direct evidence on the role of the antibodies in cytotoxicity and pathogenesis of autoimmune diseases.

## Conclusion

1. Anti-DNA autoantibodies from the sera of patients with autoimmune and lymphoproliferative diseases displayed cytotoxicity toward the tumor-cell lines HL-60 and L-929. The cytotoxic effect of anti-DNA antibodies from SLE patients was comparable to that of monoclonal anti-Fas antibody IPO-4.
2. Cytotoxic effects of SLE- and CLL-derived anti-DNA antibodies was the property of the Fab fragment, and was inhibited by preincubation with dsDNA.
3. Cytotoxic anti-DNA antibodies cross-reacted with the nuclear-matrix and plasma membrane proteins. The cross-reactivity with cellular membrane proteins may underlie the binding of cytotoxic anti-DNA antibodies to cellular receptors and initiation of signal transduction events, eventually provoking death of the target cell.
4. The DNA-hydrolyzing activity and cytotoxic activity of anti-DNA antibodies in SLE and CLL patients were correlated. DNA-hydrolyzing antibodies could presumably enter the cell and nucleus and cause DNA degradation, resulting in cellular death.

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## Discussion

*Shoenfeld*: Several studies have indicated that anti-DNA binds to cells and may be cytotoxic. In 1984 we published a monoclonal anti-DNA antibody with lymphotoxic activity. Then there was a series of papers published by Jacob, who referred to the autoantigen as LAMP—lymphocyte-associated membrane protein. Then the subject disappeared. Nobody talked about it for a long time. What I would like to see is that the antibodies do not just bind the DNA, which is just passively absorbed by the cells. Have you treated the cells with DNase to see whether binding is retained?

*Kozyr*: We are planning to treat the cells with DNase soon. When we incubate antibody with DNA, the cytotoxicity disappeared.

*Kohler*: How many cell types have you tested?

*Kozyr*: We studied two cell lines, and we plan additional cell types.

*Kohler*: What is the mechanism of cytotoxicity?

*Kozyr*: The antibodies might penetrate into the nucleus. However, many antibodies penetrate without being cytotoxic. The antibodies are complement-independent.

*Paul*: This issue of internalization of anti-DNA antibodies is obviously of great interest. The consensus seems to be that internalization is initiated on account of binding of antibodies to cross-reactive antigens on the cell surface. Mike Madaio says myosin binds anti-DNA antibodies. There is also some evidence that DNase I might bind the antibodies. Do you think the DNase activity of the antibodies is linked causally to the toxicity?

*Kozyr*: Yes, there may be a link, but we need precise experiments to outline the link.

*Paul*: Dr. Gabibov, I like the notion that the autoantibodies with catalytic activity might be anti-Ids. Is it only because it's an attractive hypothesis that you believe in it or do you have data?

*Gabibov*: The data are five years old. We proved that there was DNA-hydrolyzing activity in antiidiotypic antibody fractions. However, the anti-Id hypothesis is only one of the explanations of the DNA-hydrolyzing activity because our DNase BV04-01 is not an antiidiotypic antibody. I want to stress that BV04-01 is especially effective for single-stranded DNA, but it also works with supercoiled DNA. That is why we have to be very cautious with theory.

*Paul*: I cannot even see an experiment in which you can easily answer whether an autoantibody catalytic activity is caused by stimulation by an antibody to the enzyme. We evaluate the specificity of autoantibodies simply by titrating them against different antigens. If we get good activity, we say these antibodies are anti-DNA or anti-VIP, and so forth. The immunogens responsible for stimulation of the synthesis of these antibodies are unknown.

*Gabibov*: The second possible reason that DNA-hydrolyzing antibodies are induced during the autoimmune disease is that some unknown and unusual structure, perhaps a protein associated with a DNA becomes immunogenic. We isolated antibodies reactive with a nucleoprotein from human serum and used it as the first antibody to prepare antiidiotypes. This antiidiotypic antibody posses low-activity DNA-hydrolyzing activity. This can be the explanation.

*Schowen*: When you make antibodies against an enzyme, is there an excess of antibodies against the active-site region, or are they just random?

*Gabibov*: In my case, I do not know how first antibodies against self-enzymes are generated during the immune process, and against what region they are directed. In Daniel Thomas' work, when you induce artificial antiidiotypic antibodies, you must use a clone which inhibits the activity and is presumed to be directed against the enzyme-active site.

*Sela*: Do you see this activity with eukryotic DNA as well as the prokayotic DNA you have shown?

*Gabibov*: In the beginning, we used as substrate DNA isolated from nuclear-protein complexes from patients. There was no real specificity, so we used supercoiled DNAs.

*Sela*: Then you don't know?

*Gabibov*: I think there is activity, but it is very, very difficult to isolate supercoiled DNA from eukeryotic cells.