# IMMUNOLOGY AND MICROBIOLOGY

# Mechanisms Underlying Cytotoxicity of Anti-DNA Antibodies

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We studied mechanisms underlying cytotoxicity of catalytic anti-DNA autoantibodies or DNA abzymes isolated from the blood of patients with systemic lupus erythematosus and autoimmune mice. Experiments were performed on L929, HL-60, Raji, and K562 cells. Treatment with DNA abzymes caused internucleosomal DNA fragmentation in target cells typical of apoptosis. Direct immunofluorescence assay demonstrated that DNA abzymes can penetrate into the nucleus of target cells. The dependence of antibody-mediated cytotoxicity on the duration of incubation indicated that cell death is realized via at least 2 mechanisms: penetration of DNA abzymes into the cell nucleus followed by degradation of nuclear DNA or induction of apoptosis.

**Key Words:** *DNA abzymes; cytotoxicity; apoptosis; autoimmunity*

DNA-binding autoantibodies (AAB) can penetrate into the cell, trigger apoptosis, and display cytotoxic activity *in vitro* [6,8,14].

Previous studies demonstrated catalytic and cytotoxic properties of anti-DNA AAB isolated from the blood of patients with systemic lupus erythematosus (SLE) and autoimmune MRL-lpr/lpr mice [12]. Here we studied the mechanisms of cytotoxicity mediated by anti-DNA AAB and their role in cell death.

## MATERIALS AND METHODS

Blood samples from patients with SLE ($n$=40) containing DNA abzymes were obtained from the collection of serum samples (Institute of Rheumatology, Russian Academy of Medical Sciences; Moscow Regional Clinical Institute). HL-60, Raji, L929, and K562 cells were obtained from the American Type Culture Collection. Inbred BALB/c and MRL-lpr/lpr mice were kept under sterile conditions. Salts, N-acetyl-YVAD-CHO, and materials for cell culturing were obtained from Life Technologies and Sigma. Purification of DNA-binding AAB, estimation of their purity, and measurements of antibody (AB) activity were performed as described previously [12]. Cytotoxicity of AB was evaluated using L929, HL-60, Raji, and K-562 target cells [11]. After incubation with AAB, dead cells were counted using trypan blue exclusion and MTT test. For inhibition of ICE protease YVAD-CHO (Sigma) was added to L929 cells before incubation with AAB. The buffer served as the control. Dead cells were counted 1, 2, 3, 4, 5, 18, 24, and 30 h after the start of incubation.

Raji cells were incubated with AAB binding and not binding DNA for 3 h. Control samples contained no AAB. Cells incubated with anti-Fas IPO-4 AAB served as the positive control. The number of apoptotic cells was estimated by cell binding with annexin V and elimination of propidium iodide. The cells were incubated with FITC-annexin V (Immunotech) and propidium iodide as described elsewhere [11]. The samples were studied by flow cytometry. The data were analyzed on a MultiGraph device (Coulter).

Penetration of AAB into living cells and nuclei was assayed by immunofluorescence microscopy using FITC-AAB conjugates [11]. Viable Raji cells were incubated with FITC-AAB at 4°C for 1 h. Control

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cells were incubated with FITC-bovine serum albumin (BSA), and free IgG were then removed. AAB were visualized under a Leitz Dialux 22 fluorescence microscope.

DNA fragmentation was evaluated by agarose gel electrophoresis [7,12]. HL-60 cells were incubated in the presence of DNA abzymes, tumor necrosis factor-α (TNF-α), or anti-Fas receptor IPO-4 AAB inducing apoptosis for 48 h. The cells were then centrifuged and lysed. DNA was treated with RNase A and proteinase K, subjected to electrophoresis in 1.5% agarose gel, and stained with ethidium bromide.

## RESULTS

Electrophoresis of DNA isolated from target cells after incubation with cytotoxic anti-DNA AAB from patients with SLE revealed internucleosomal fragmentation of DNA typical of apoptosis (Fig. 1). Similar results were obtained after incubation of cells with TNF-α or anti-Fas IPO-4 AAB (markers of apoptosis).

Four hours after incubation with cytotoxic anti-DNA AAB at least 20% target Raji cells were stained with annexin, which confirmed the involvement of AAB in the early stage of apoptosis. Under these conditions, 4% Raji cells were stained with propidium iodide. Double staining with annexin and propidium iodide was found in 8.3% cells, which confirmed apoptosis (Fig. 2). IPO-4 AAB were effective in 35, 1.5, and 6.8% cells, respectively. Less than 5, 1, and 4% control cells were stained with annexin, propidium iodide, and annexin+propidium iodide, respectively.

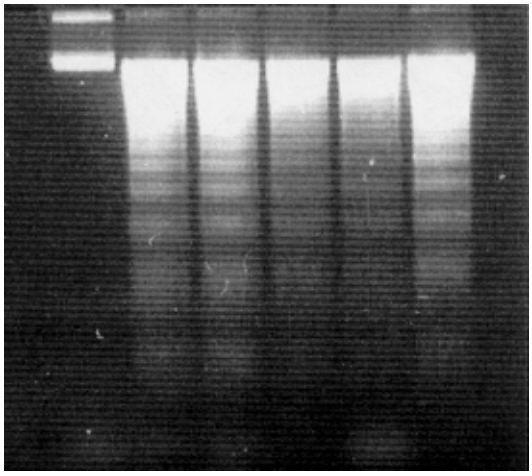


**Fig. 1.** Fragmentation of target cell DNA caused by DNA-binding autoantibodies (AAB). Incubation in the absence (*1*) or in the presence of anti-DNA AAB from a patient with systemic lupus erythematosus (0.1 μM, *2*), anti-DNA AAB from MRL-lpr/lpr mice (0.1 μM, *3*), agonist AB APO-4 (0.1 μM, *4*), and 0.1 (*5*) or 0.01 μM tumor necrosis factor-α (*6*).

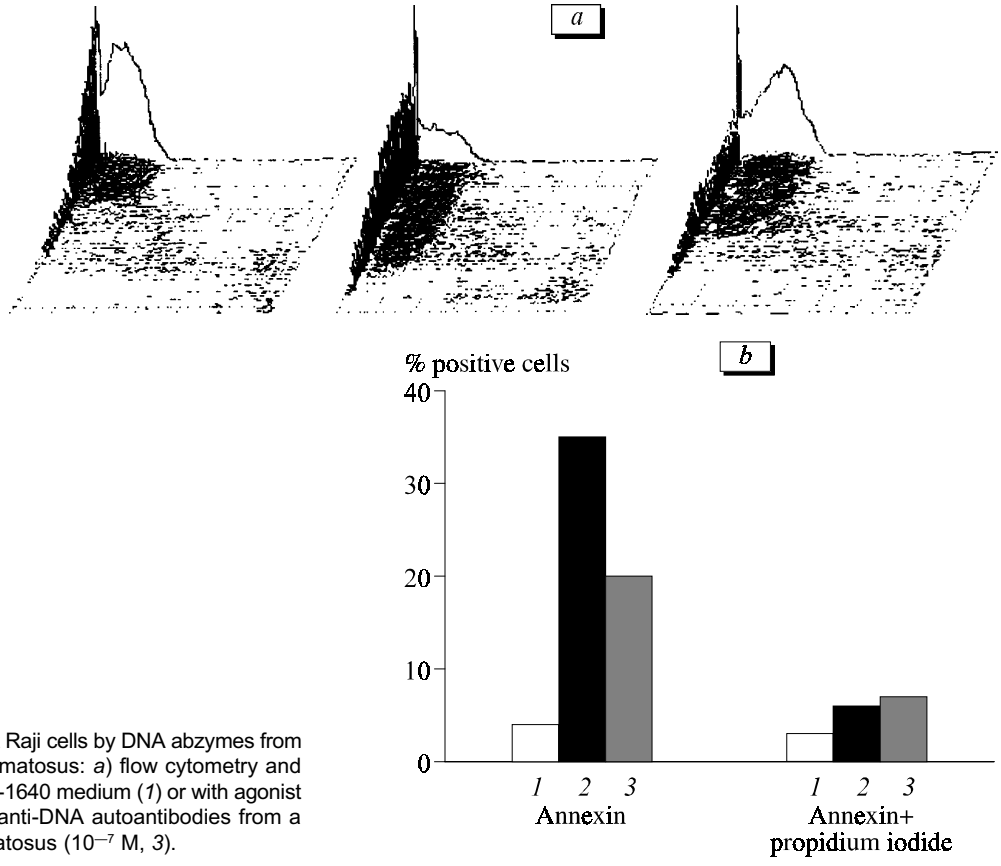


**Fig. 2.** Induction of apoptosis in target Raji cells by DNA abzymes from a patient with systemic lupus erythematosus: *a*) flow cytometry and *b*) averaged data. Incubation in RPMI-1640 medium (*1*) or with agonist anti-Fas AB IPO-4 ($10^{-7}$ M, *2*) and anti-DNA autoantibodies from a patient with systemic lupus erythematosus ($10^{-7}$ M, *3*).

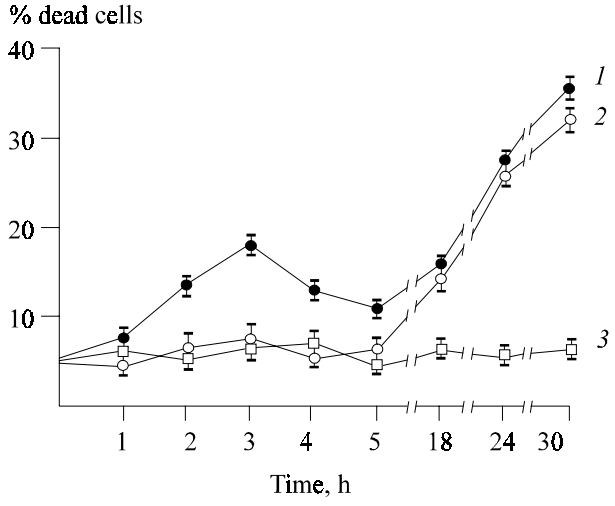


**Fig. 3.** Dynamics of cytotoxicity induced in target L929 cells by DNA abzymes from a patient with systemic lupus erythematosus. Incubation without YVAD-CHO (*1*), in RPMI-1640 medium (*2*), and in the presence of YVAD-CHO (*3*).

Similar results were obtained in experiments with DNA abzymes isolated from autoimmune MRL-lpr/lpr mice. Thus, DNA abzymes from patients with SLE and autoimmune mice can induce apoptosis.

Two peaks of cell death were identified after incubation of target L929 cells with 10 nmol anti-DNA AAB from patients with SLE (Fig. 3). Peak 1 was observed after 3-h incubation (20% dead cells); then the percentage of dead cells decreased to 4-6%. Peak 2 was observed after 18-48-h incubation (32-37% dead cells).

To compare the dynamics of cell death induced by anti-DNA AAB, target cells were preincubated with the specific caspase I inhibitor YVAD-CHO that blocks caspase-dependent apoptosis [7]. This treatment abolished peak 1, but not peak 2 (18-30-h incubation; Fig. 3). These results suggest that cell death is realized via at least 2 mechanisms mediated by cytotoxic anti-DNA AAB.

Similar dynamics of cytotoxicity was observed after incubation of target cells with DNA abzymes obtained from 4-month-old MRL-lpr/lpr mice at the peak of SLE-like syndrome. Thus, caspase-dependent apoptosis typical of the early cytotoxic effects of DNA abzymes on target cells is not the only mechanism of their cytotoxicity. Discrete peaks of cell death observed in the dynamics of cytotoxicity indicate the existence of different mechanisms underlying activation of apoptosis or transduction of regulatory signals.

The interaction of AAB binding and not binding DNA with target Raji cells was assayed by direct fluorescence. The cells were stained with FITC-labeled AAB and incubated at 37°C for 1 h. Anti-DNA AAB interacted with the plasma membrane. The cell and cytoplasm were stained in 5% cells, which indicated damage to the plasma membrane and cell death. One hour after incubation 30-50% cells contained AAB in the nucleus, while plasma membrane was stained only in 20% cells. We found no differences between penetration of anti-DNA AAB possessing and lacking cytotoxic properties into living cell and nucleus. AAB not binding DNA had no effect on target cells. The nucleus and cell membrane did not produce fluorescence signals after incubation of target cells with FITC-BSA.

Thus, anti-DNA AAB penetrate into living cell and nucleus and produce cytotoxic and hydrolyzing effects [1,9]. However, not all AAB have specialized functional sites [2,9]. We studied only catalytic and cytotoxic properties of DNA-binding AAB obtained from patients with systemic autoimmune diseases and autoimmune mice. This is related to the existence of various AAB in these pathologies [5,7]. SLE and SLE-like syndrome accompany 35-65% clinical and experimental models characterized by the presence of cytotoxic anti-DNA AAB.

Our findings on suppressed cytotoxicity of AAB molecule with preserved cytotoxic properties of the corresponding Fab-fragment suggest a complement-independent mechanism of cell death induced by anti-DNA AAB [1]. This assumption is confirmed by the fact that synthetic AB against Fas and TNF-α receptors can simulate cytotoxic properties of natural ligands [3].

Discreteness in the asynchronous culture is probably associated with the heterogeneity of cells that undergo apoptosis in various stages of the cell cycle [6]. In our experiments cytotoxic anti-DNA AAB intensively penetrated into the cells. However, AAB did not enter 10-20% target cells. Hence, the interaction of AAB with these cells depended on the phase of cell cycle. We hypothesize that induction of the Fas-independent pathway of cell death is associated with peak 1. Therefore, the mechanisms of immediate (3 h) and delayed (18-48 h) AAB-mediated cell death are different.

Treatment of target cells with cytotoxic AAB stimulated binding of annexin to the cell surface. It was reported that annexin reacts with phosphatidylserine in the plasma membrane at the early stages of apoptosis. However, staining with propidium iodide indicates disintegration of the nuclear membrane and degradation of nuclear DNA. These processes and internucleosomal DNA fragmentation indicate that cell death (peak 1) induced by anti-DNA AAB is mediated by the apoptotic mechanism [4].

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