Natural hidden antibodies reacting with DNA or cardiolipin bind to thymocytes and evoke their death

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Abstract Both free and hidden natural antibodies to DNA or cardiolipin were obtained from immunoglobulins of a normal donor. The free antibodies reacting with DNA or cardiolipin were isolated by means of affinity chromatography. Antibodies occurring in an hidden state were disengaged from the depleted immunoglobulins by ion-exchange chromatography and were then affinity-isolated on DNA or cardiolipin sorbents. We used flow cytometry to study the ability of free and hidden antibodies to bind to rat thymocytes. Simultaneously, plasma membrane integrity was tested by propidium iodide (PI) exclusion. The hidden antibodies reacted with 65.2 ± 10.9% of the thymocytes and caused a fast plasma membrane disruption. Cells (28.7 ± 7.1%) were stained with PI after incubation with the hidden antibodies for 1 h. The free antibodies bound to a very small fraction of the thymocytes and did not evoke death as compared to control without antibodies. The possible reason for the observed effects is difference in reactivity of the free and hidden antibodies to phospholipids. While free antibodies reacted preferentially with phosphotidylcholine, hidden antibodies reacted with cardiolipin and phosphotidylserine.

Key words: Flow cytometry; Cell death; Natural antibody; Hidden antibody; Antibody to DNA; Antibody to cardiolipin; Rat thymocyte

1. Introduction

In recent years the problems of natural antibodies (origin, functions, activity regulation) [1,2] have attracted considerable interest.

Natural antibodies are an obligatory component of normal immunity. They have been found in blood plasma of healthy individuals and animals. In addition, up to 30% of hybridomas that can be obtained from healthy individuals produce natural antibodies [3].

The most characteristic feature of natural antibodies is their polyspecificity. They react with DNA, phospholipids, proteins of cytoskeleton and proteins of plasma membrane. Perhaps, the reason for polyspecificity is the ability of natural antibodies to react with various antigens via electrostatic interaction [4–6].

In our previous studies it was shown that a significant amount of antibody to DNA and to cardiolipin is in a hidden state and its activity is displayed only after treatment by ion-exchange chromatography on QAE-Sephadex or gel filtration at pH 4.5 [5]. These data have been confirmed by other researchers [7,8].

Functions of natural antibodies are widely discussed. However, there are no data regarding the possible functions of hidden antibodies described in our studies. It is known that anti-DNA antibodies (derived from patients with systemic lupus erythematosis) is able to interact with various types of membranes and evoke the death of mesangial and endothelial cells [9–11]. Taking this into account, in a number of features, hidden antibodies to DNA are similar to those known for lupus antibodies. Thus, it would be interesting to investigate the analogous properties of hidden antibodies.

In the present study, the ability of hidden antibodies to react with T-cells and evoke their death is investigated.

2. Materials and methods

10% Normal human γ-globulin (γG) solution produced for intramuscular administration (Moscow Research Institute of Epidemiology and Microbiology; the technological procedure of manufacturing involves the alcohol fractionation of serum proteins according to Cohn), 'Sandoglobulin' (normal human immunoglobulins for intravenous infusion; Sandoz Pharm. Ltd., Switzerland) were used as the source of antibodies.

2.1. Antibody isolation

γG preparations were diluted in 0.1 M phosphate-buffered solution (PBS; 10–12 mg/ml), recirculated for 16–18 h on an affinity column containing DNA or cardiolipin sorbents and the fraction of absorbed γG (free antibodies) was eluted with 2 M NaCl. The procedure was repeated several times until the complete removal of antibodies which bound DNA or cardiolipin from γG. The depleted γG was dialyzed against 0.01 M potassium phosphate buffer (pH 7.3) and mixed with QAE–Sephadex A-50 beads (1 ml of gel/5 mg protein) in the same buffer for 1.5 h. The slurry was packed into a column and two fractions were eluted with 0.01 M potassium phosphate buffer and with 0.5 M NaCl. Antibodies to DNA or to cardiolipin from these fractions (so-called hidden antibodies) were isolated on the corresponding affinity columns. Antibodies were dialyzed against 0.01 M PBS and concentrated. Protein concentration was determined by Bradford's assay.

Interaction of the antibodies with different phospholipids was assessed by ELISA as described [12].

2.2. Thymocyte isolation, incubation and staining

Cells were isolated from thymus of young rats (3–4 weeks old) by rubbing through a nylon mesh and thereafter washing with 0.01 M PBS, pH 7.2. The thymocytes ($5 \cdot 10^5$ cells) were incubated with 20 μg of either free or hidden antibodies in 1 ml of PBS containing 0.5% bovine serum albumin (BSA, Sigma) for 1 h at 37°C. To determine the dependence of binding and cell death on antibody concentration, we used 1–40 μg/ml of free or hidden antibodies. After treatment, cells were washed 2 times with PBS/BSA and then incubated with FITC-

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Abbreviations: PI, propidium iodide; γG, γ-globulin; PBS, phosphate-buffered solution; OD, optical density

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labeled antibodies to human IgG (Sigma Chemical Co., USA) for 1 h at 37°C. Then the thymocytes were washed, resuspended in PBS and stained with PI (Sigma Chemical Co., 40 μg/ml) for 10 min at room temperature before beginning flow cytometry.

To determine the dependence of cell death on incubation time with antibodies, thymocytes were incubated with 20 μg of antibodies for 5 min to 3 h at 37°C, then washed with PBS and stained with PI for 10 min.

To determine DNA fragmentation, after 1 h treatment with free or hidden antibodies (20 μg/5·10^5 cells) cells were lysed with 0.2% Triton X-100, 4 mM Tris-HCl, 1 mM EDTA (pH 7.5) for 5 min. After centrifugation at 6000×g for 5 min, the fraction of fragmented (supernate) DNA was evaluated fluorimetrically using Hoechst 33258 (2 μM).

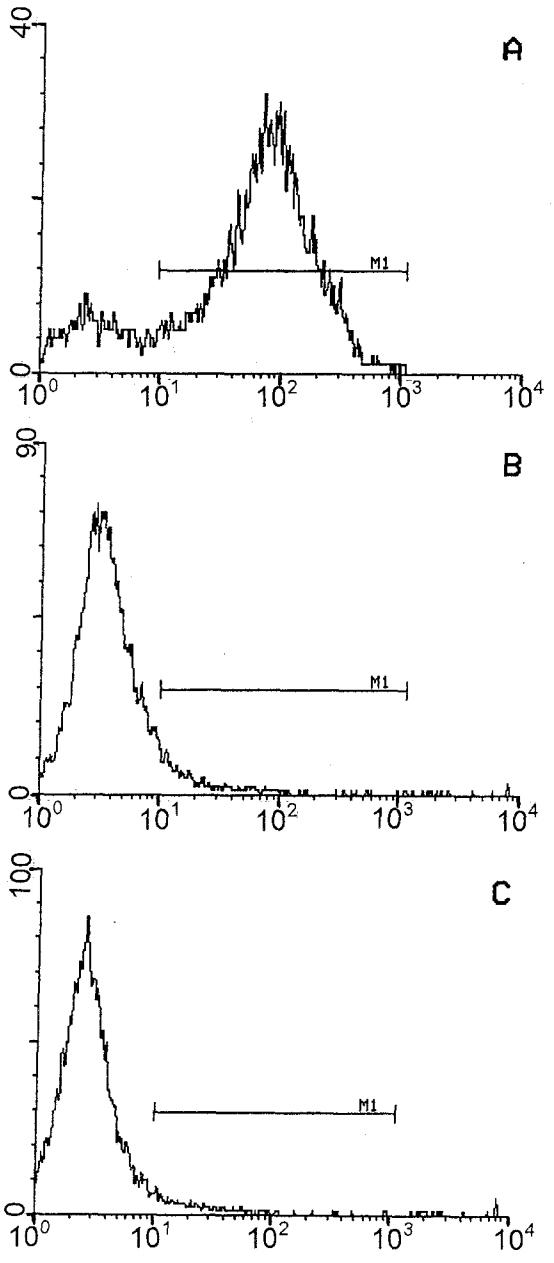

Fig. 1. Typical histograms of the binding of hidden antibodies (A), free antibodies (B) to the thymocytes and control sample incubated only with FITC-labeled antibodies to human Ig (C). Abscissa: FITC fluorescence intensity; ordinate: number of cells.

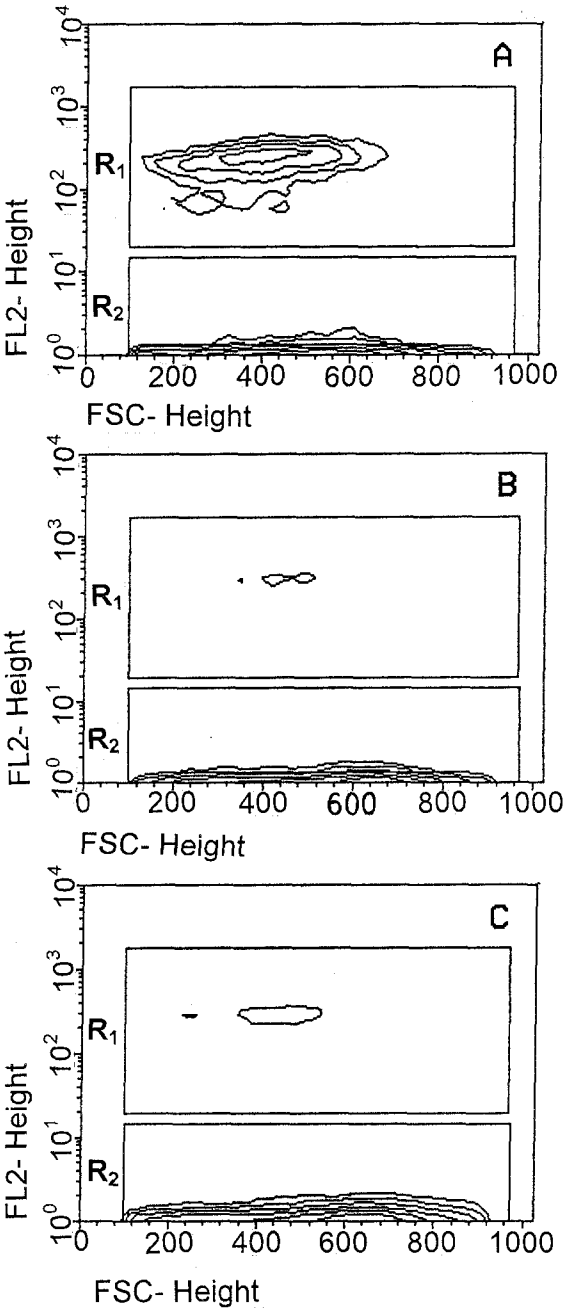

Fig. 2. Ordinate: PI fluorescence in thymocytes after treatment with hidden antibodies (A), free antibodies (B) and in control sample without primary antibodies (C). Abscissa: Forward scatter values.

2.3. Flow cytometry

Samples were analyzed on FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, CA) equipped with argon ion laser Enterprise 621 (Coherent, CA) operating at 488 nm (150 mW). FITC and PI fluorescences were collected with 530/30 and 582/42 nm filters, respectively. Twenty-thousand events were analyzed for each sample. Data acquisition and analysis were performed with a Hewlett-Packard 340 computer using 'Lysys II' software (Becton Dickinson).

After gating on a cytogram of light scatter to exclude cell clumps and debris, a cytogram of PI fluorescence versus forward light scatter was recorded to determine the fraction of cells which included PI due to loss of plasma membrane integrity. The fraction of cells binding antibodies and mean fluorescence intensity of labeled cells were estimated using FITC fluorescence histograms after exclusion of clumps and debris.

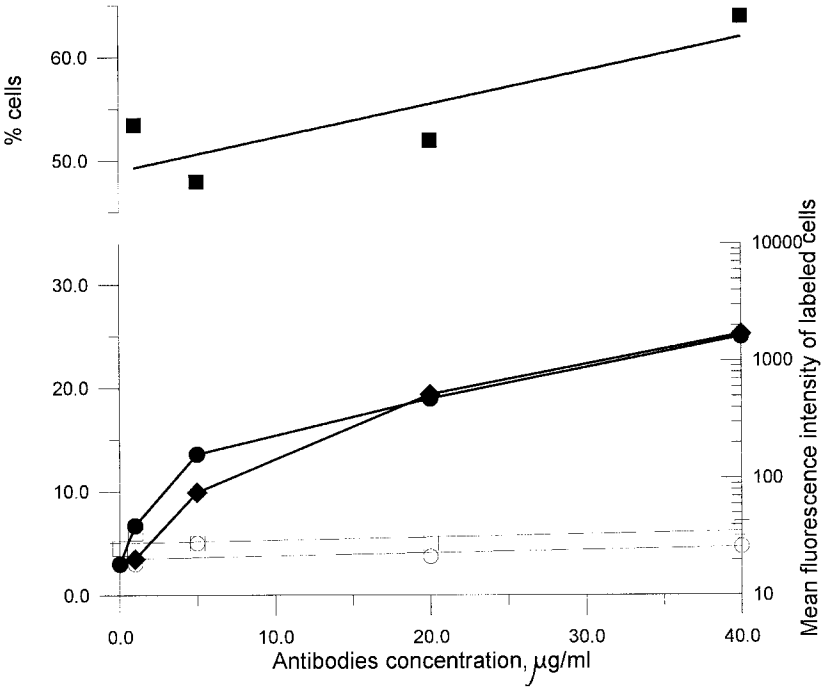

Fig. 3. Dependence of number of PI-positive cells (●), cells binding antibodies (■) and mean fluorescence intensity of labeled cells (◆) on the hidden antibody concentration. Dependence of number of PI-positive cells (○) and cells binding antibodies (□) on the free antibody concentration.

3. Results

The intensity of binding of the free antibody to DNA obtained from immunoglobulin preparations and the hidden antibody to the thymocytes is shown in Fig. 1 as histograms. 70% of thymocytes bind the hidden antibodies and fall into region M1. Not more than 5% of cells fall into M1 after treatment with the free antibodies or in the control sample without primary antibodies (Table 1).

Fig. 2 is a contour plot which allows determination of the fraction of cells stained with PI. The cells localized to R1 region were characterized by bright PI fluorescence and referred to as dead. No effect of free antibodies on cell death was observed, whereas incubation of thymocytes with hidden antibodies brought about pronounced elevation in the number of dead cells (Table 1). We did not observed DNA fragmentation after treatment with hidden antibodies in the fluorometric test with Hoechst 33258 (data not shown).

We also studied the dependence of the effects on antibody concentration (1–40 μg/ml). The results of a typical experiment are shown in Fig. 3. Increase in concentration of hidden antibodies did not appreciably change the fraction of cells binding antibody, while a significant increase in fluorescence of labeled cells was observed. Augmentation of PI-positive cell number correlated with the mean fluorescence intensity of labeled cells ($r = 0.98$).

Dependence of the cell death on incubation time with antibodies to DNA is shown in Fig. 4. Hidden antibodies evoked fast plasma membrane disruption in 14.0% of the thymocytes during 30 min; free antibodies had no effect.

Thus, an essential distinction was found between free and hidden antibodies to DNA in their ability to bind to thymocyte surface as well as to cause their death. Similar results were observed with affinity purified hidden antibodies to cardiolipin (data not shown).

ELISA data in Table 2 show that there is a difference in the ability of free and hidden antibodies to react with phospholipids. Free antibodies reacted preferentially with phosphotidylcholine whereas hidden antibodies reacted with cardiolipin and phosphotidylserine.

4. Discussion

Free antibodies reacting with DNA or cardiolipin were isolated by means of affinity chromatography. Antibodies occurring in a hidden state were disengaged from the depleted im-

Table 1
Summary of the flow cytometric analyses of thymocytes treated with free and hidden antibodies (20 μg/ml)

Antibodies	Fraction of cells binding antibodies (% ±SD)	Fraction of PI-positive cells (% ±SD)
Control without primary antibodies	–	3.2±0.9
Free antibodies	5.6± 2.2	3.3±0.9
Hidden antibodies	65.2±10.9	28.7±7.1

Results shown are averages of five experiments.

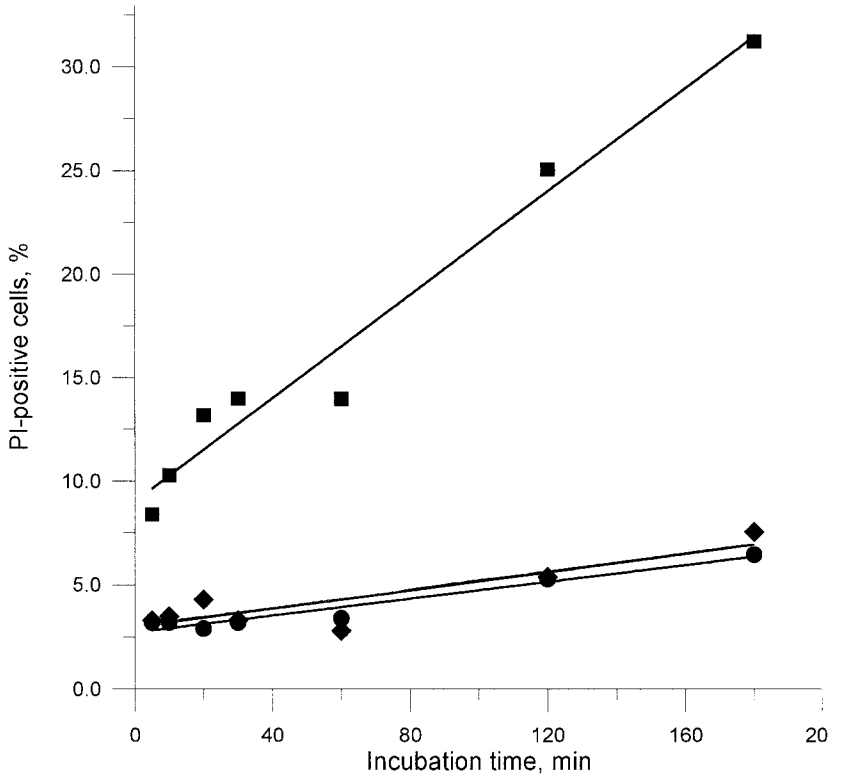

Fig. 4. Dependence of PI-positive cell number on the time of incubation with hidden antibodies (■), free antibodies (♦) to DNA and in control samples without primary antibodies (●).

munoglobulins by ion-exchange chromatography and were then affinity isolated on DNA or cardiolipin sorbents. The amount of hidden antibodies was 4–5-fold greater than that of the free antibodies. It was shown that only hidden antibodies interacted with thymocytes and caused the death of their pronounced fraction.

A form of cell death evoked by hidden antibodies is not yet definitively clear. It is known that a critical event in apoptosis is the increase in cytosolic Ca^{2+} due to internal mobilization and influx of extracellular Ca^{2+} [13,14]. Preliminary data were obtained regarding a profound elevation of the membrane permeability towards ^{45}Ca (a 2-fold increase in the initial rate of Ca^{2+} uptake) observed within the same time span (paper in preparation). However, within 1 h when we clearly observed the PI-detectable death of a significant number of cells, there was no DNA fragmentation characteristic of apoptosis.

Investigation of free and hidden Ab properties revealed that the ability to react with phospholipids is essentially diverse; free antibodies reacted preferentially with phosphotidylcholine whereas hidden antibodies reacted with cardiolipin and phosphotidylserine. Apparently, the most important role in epitope formation is determined by the presence of a positive or negative charge.

It is significant that the killer antibodies causing cell death exist in the hidden state that may indicate the presence of some system which prevents the cytotoxic effect of this antibody. Appearance of killer antibodies in a free state may result from an inability of serum to suppress its activity. We have found that the appearance of antibodies with similar properties may occur under traumatic or burn shock conditions [15].

Hidden antibodies to DNA or to cardiolipin binding to the thymocytes and causing their death is likely to play an important role in maintaining homeostasis. Investigation of the cytotoxic effect mechanisms of hidden antibodies is in progress.

Table 2
Interaction of the free and hidden antibodies (50 μg/ml) with phospholipids (OD_{492})

Antibodies	Cardiolipin	Phosphotidylserine	Phosphotidylcholine	Cardiolipin/phosphotidylcholine	Phosphotidylserine/phosphotidilcholine
Free antibodies to DNA	0.37	0.66	0.95	0.39	0.69
Hidden antibodies to DNA	1.53	1.33	0.36	4.25	3.69
Free antibodies to cardiolipin	0.11	0.12	0.15	0.73	0.80
Hidden antibodies to cardiolipin	0.65	0.87	0.10	6.50	8.70

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