

The effects of anti-idiotypic antibody on antibody production and apoptosis of anti-dsDNA antibody producing cells

C.H. Lee, C.H. Suh, J. Lee, Y.T. Kim¹, S.K. Lee

Division of Rheumatology, Department of Internal Medicine, Institute for Immunology and Immunological Disease, BK 21 Project for Medical Sciences, Yonsei University College of Medicine, Seoul; ¹Department of Microbiology, Ajou University College of Medicine, Suwon, Korea.

Abstract Objective

Systemic lupus erythematosus is an autoimmune disease characterized by the production of anti-dsDNA antibody. Because the titer of anti-dsDNA antibody is correlated with disease severity, especially in lupus nephritis, controlling anti-dsDNA antibody production is important in the treatment of SLE. There are many regulatory mechanisms of autoantibody production; one of these is the interaction between idiotype and anti-idiotypic antibody (anti-Id). The purpose of the present study was to assess the effect of anti-Id on anti-dsDNA antibody production and apoptosis and to study the mechanism of anti-Id induced apoptosis.

Methods

After anti-dsDNA antibody producing hybridomas were treated with anti-Id, we checked the amount of anti-dsDNA antibody production, the rate of transcription, cellular proliferation, and apoptosis. Also, after treatment with anti-oxidant (N-acetyl-L-cysteine), phorbol esters with calcium ionophore and corticosteroids, we compared their effect on apoptosis with anti-Id.

Results

Two types of anti-dsDNA antibody producing hybridomas (G1-2, γ and κ chains; M2-10, μ and κ chains) were treated with anti-Id and it was found that: (1) the amount of anti-dsDNA antibody production decreased; (2) the rate of transcription and cellular proliferation did not decrease; and (3) the level of apoptosis increased. The two cells expressed Fas and Fas-ligand, and the Fas of G1-2 was functional but that of M2-10 was not. The treatment of these cells with anti-Id resulted in no change in Fas-ligand and Bax expression, but the expression of Bcl-2 was decreased. In addition, treatment with antioxidant (N-acetyl-L-cysteine) inhibited anti-Id-induced apoptosis in G1-2 and M2-10. Phorbol esters with calcium ionophore also inhibited anti-Id induced apoptosis in M2-10. Corticosteroids induced apoptosis in both cells and showed similar results with anti-Id induced apoptosis.

Conclusion

The anti-Id suppressed the production of anti-dsDNA antibody in two cells by inducing apoptosis, as did prednisolone. Furthermore, Bcl-2, oxygen-free radicals and protein kinase C might be involved in the induction of apoptosis by anti-Id.

Key words

Anti-dsDNA antibody, anti-idiotypic antibody, apoptosis, corticosteroid.

Chan-Hee Lee, MD, PhD; Chang-Hee Suh, MD, PhD; Jisoo Lee, MD, PhD; Young Tai Kim, PhD; Soo-Kon Lee, MD, PhD. Young Tai Kim, PhD.

Please address correspondence and reprint requests to: Dr. Soo-Kon Lee, Division of Rheumatology, Department of Internal Medicine, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul, Korea.

E-mail: sookonlee@yumc.yonsei.ac.kr

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Abbreviations: SLE: systemic lupus erythematosus; anti-Id: anti-idiotypic antibody; BCR: B cell receptor; Ig: immuno-globulin; FasL: Fas ligand; CS: corticosteroids; PARP: poly (ADP-ribose) polymerase.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of various autoantibodies. Antibodies to ds-DNA play an important role in the diagnosis of SLE. The titer of anti-dsDNA antibody is frequently associated with increased disease activity, especially in lupus nephritis (1-3).

Anti-idiotypic antibodies (anti-Id) recognizing the variable regions of antibody may regulate the expression of idiotype determinants on B cell surfaces and could thus play a key role in the induction of self-tolerance (4, 5). Thus, interest has focused on anti-Id for the treatment of autoimmune disease. There are some reports that describe the successful manipulation of autoantibody production by anti-Id in several autoimmune diseases. Especially in animal model of SLE, treatment with monoclonal anti-Id showed a reduction of the titer of anti-dsDNA antibody and diminished clinical manifestations (6-9). Anti-Id may be thus helpful in the treatment of SLE, but its mechanism has not been fully clarified. Binding of B cell receptor (BCR) to anti-immunoglobulin (anti-Ig) antibody is thought to show the same effects as anti-Id. When the IgM antigen receptor was crosslinked with polyclonal anti-IgM antibody in mouse B lymphocytes, the amount of antibody production decreased. These B lymphocytes did not show reduced proliferation but progressed toward apoptosis (10). Furthermore, when B lymphocytes in Peyer's patches in sheep were treated with anti-Ig antibodies, apoptosis was increased (11).

Thus, anti-Ig antibody is thought to play an important role in the induction of apoptosis. Unlike necrosis, which is associated with increasing permeability of the plasma membrane, apoptosis is characterized by DNA fragmentation, membrane blebbing, and chromatin condensation (12). Apoptosis can be induced actively through the ligation of specific receptors such as Fas, or passively through the lack of essential survival signals such as IL-2 and interferon-3, which act by upregulating the expression of antiapoptotic molecules

such as Bcl-2 (13,14). The study of Renschler *et al.* (15), which was conducted using lymphoma cells, was until recently the only report on apoptosis relating to anti-Id. Since these lymphoma cells go through spontaneous apoptosis *in vitro*, their study attempted to verify apoptosis due to anti-Id by measuring extracellular acidification under laboratory conditions. Corticosteroids (CS) are immunosuppressive drugs that are widely used to treat SLE and decrease anti-dsDNA antibody titer (16). It is known that apoptosis can be induced by CS. The mechanism of apoptosis induced by CS is not clear, but many biochemical and molecular changes have been shown to occur in lymphocytes in response to CS (17-19). We produced two anti-dsDNA antibody producing hybridoma from the MRL-*lpr/lpr* (MRL/*lpr*) mouse, an animal model of SLE, that naturally produces several autoantibodies including anti-dsDNA antibody (20, 21), and studied the effect of anti-Id on antibody production and apoptosis. We also determined the CS effect on these cells.

Materials and methods

Monoclonal anti-dsDNA antibody producing cells

We obtained hybridoma cells from the fusion of SP2/0 myeloma cells and spleen cells of a 26-week-old MRL/*lpr* mouse with 50% polyethylene glycol 4000 (Gibco BRL, Gaithersburg, MD) solution. The hybridoma cells were cultured in HAT medium supplemented with 20% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C under a 5% CO₂ environment. Supernatants of hybridoma culture fluid were screened for anti-dsDNA by ELISA as described previously (22, 23). We selected cells which produced anti-dsDNA antibodies and cloned them twice by limiting dilution with DMEM (Sigma, St. Louis, MO) containing 10% FBS. We used an isotyping kit (Pierce, Rockford, IL) to determine the isotypes of anti-dsDNA monoclonal antibody producing cells, which were G1-2 (g and k chain production) and M2-10 (m and k chain production) cells. After bulk culture of

these cells, we purified monoclonal antibodies using affinity chromatography with a DNA agarose column (Gibco BRL, Gaithersburg, MD). The concentration of antibodies was measured by absorbance at 280 nm using a spectrophotometer (Bio-Rad, Hercules, CA). Purified antibodies were detected by SDS-PAGE and western blotting.

Anti-Id production

The same amount of purified anti-ds DNA antibody (1 mg/ml) from G1-2 cells culture supernatants and complete Freund's adjuvant (Sigma, St. Louis, MO) were mixed and inoculated into a rabbit and those of M2-10 cells into another rabbit. Rabbits received booster shots in the same manner 3 weeks later, and then twice afterwards at one-week intervals, and were bled one week after the last boosting. Sera collected from blood were tested with ELISA for the documentation of reactivity with anti-dsDNA antibody. The immunoglobulin fraction of the sera that was prepared with 50% ammonium sulfate was verified by western blotting, and affinity purified on an agarose column conjugated with normal mouse-IgG (or CNBr-Sepharose column conjugated with normal mouse-IgM in M2-10) for removal of fraction that bind with Fc portion of mouse immunoglobulin (24). Anti-Id to IgG (or IgM) anti-DNA antibody were obtained by passage through a protein A column (or anti-rabbit IgM agarose column in M2-10) of purified immunoglobulin. Anti-Id from the sera of rabbits were verified for specificity to with ELISA and used in the present study. For the control group, Ig separated from the extracted pre-inoculation serum was used.

Anti-dsDNA antibody ELISA

Anti-Id or normal Ig was added to the G1-2 and M2-10 cells (1 ml of 5×10^4 cells/ml) at dilutions of 5, 10, 100, and 1,000-fold. After culturing for 24, 48 and 72 hrs in 24-well plates, ELISA was performed. In 96-well plates coated with calf thymus dsDNA (Sigma, St. Louis, MO), 0.1 ml of each cell culture supernatant was added. Alkaline phosphatase conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO) at a dilu-

tion of 1:10,000 was applied to the plate, and then p-nitrophenyl phosphate (Sigma, St. Louis, MO) was added as substrate. The plates were read at 405 nm using ELISA plate reader (Bio-Rad, Hercules, Ca). Optical density (OD) values were determined as the mean of triplicate readings.

Measurement of cell proliferation

G1-2 and M2-10 cells (0.1 ml of 5×10^4 cells/ml) were placed in 96-well round bottom plates and treated with anti-Id or normal Ig for 30 hrs. One microcurie of ^3H thymidine (6.7 Ci/miMole, New England Nuclear, Boston, MA) was added to each well. The cells were harvested onto glass-fiber filters after an additional 18 hours of culture. The levels of cell proliferation were determined by measuring radioactivity with a scintillation counter (Pharmacia LKB, Uppsala, Sweden).

Northern blot

In order to compare the transcription rates, anti-Id diluted 10-fold was added to G1-2 and M2-10 cells (20 ml of 5×10^4 cells/ml) and cultured for 48 hrs. Total RNA was separated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and electrophoresed. The RNA bands were moved to a nylon membrane and hybridization with Cg, Cm and Ck probes were performed. After washing, the bands were exposed for several hours on an imaging plate (BAS-MP 2040S, Fuji, Kanagawa, Japan) and the intensity of each band was measured with a phosphor imager (BAS-2500, Fuji, Kanagawa, Japan). The band's intensity was compared with the control group after performing internal standardization with the intensity of the b-actin band.

Flow cytometric analysis

Fas and FasL. One ml of G1-2 or M2-10 cells (5×10^4 cells/ml) was cultured in a 24-well plate for 3 hrs, and then the cells were collected. After adding FITC conjugated anti-mouse Fas antibody (mouse, monoclonal IgG, Pharmingen, San Diego, CA) at a concentration of $1 \text{ ng}/10^6$ cells, the cells were left in the dark at 4°C for 30 hours. The cells were washed again with PBS and ana-

lyzed using a FACStar Plus (Becton Dickinson, Mountain View, CA).

For FasL, the G1-2 and M2-10 cells were cultured with anti-Id or prednisolone for 2, 4, 6, 14 and 22 hrs and R-phycoerythrin conjugated anti-mouse FasL antibody (mouse, monoclonal IgG, Pharmingen, San Diego, CA) was added at $4 \text{ ng}/10^6$ cells.

Measurement of apoptosis. After placing 3 ml of G1-2 and M2-10 cells in a 6-well plate and adding anti-Id or normal Ig diluted 10-fold, the plates were cultured for 2, 4, 6, 8, 24 and 48 hours. After collecting the cells, annexin V-FITC incubation reagent ($10 \times$ binding buffer, propidium iodide, annexin V-FITC, Trevigen, Gaithersburg, MD) was added and the wells were stored at room temperature in the dark. After 15 minutes, binding buffer was added and analyzed with FACStar Plus. After treating with anti-mouse Fas antibody (Jo-2, hamster, monoclonal IgG, Pharmingen, San Diego, CA) at various concentrations ($0.01 - 50 \text{ ng/ml}$) for 3, 5, 7 and 22 hrs, the cells were analyzed. To determine whether apoptosis was induced by CS, various concentrations of prednisolone (Upjohn, Hwasung, Korea) were added to the cells and cultured for 3 and 22 hrs. The cells were analyzed with FACStar Plus.

To evaluate the role of oxygen-free radicals and protein kinase C in apoptosis induced by anti-Id or prednisolone, N-acetyl-L-cysteine (Sigma, St. Louis, MO) or calcium ionophore A23187 (Sigma, St. Louis, MO) and phorbol myristate acetate (Sigma, St. Louis, MO) was added to each anti-Id or prednisolone treatment. The cells were cultured for 4 and 22 hrs and analyzed. The results were compared with those of anti-Id alone or prednisolone alone.

DNA electrophoresis

Seven ml of G1-2 and M2-10 cells were placed in a 25 cm^2 flask and anti-Id or prednisolone was added. This mixture was cultured for 5 and 7 hrs and the cells were collected. To the cell precipitate, TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) were added and left for 30 minutes at 4°C . After

centrifuging at 13,000 rpm for 15 minutes, the supernatant was collected and added with ethyl alcohol and 5 M NaCl, and the mixture was left over-night at -20°C. After centrifugation, TE buffer and RNase were added to react at 37°C for 1 hour. Protein kinase was added and incubated at 37°C for more than 2 hours and electrophoresed on 1.8% agarose gel. The bands were compared with the control.

Western blot

Cell culture and protein preparation. Anti-Id was added to 7 ml of G1-2 and M2-10 cells at a ratio of 1: 10 in a 25 cm² flask and the cells were cultured according to the time schedule. The cells were mixed with 50 ml lysis buffer (70 mM b-glycerophosphate pH 7.2, 0.1 mM Na-Vanavate, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, and 0.2 mM phenylmethylsulfonylfluoride) and left on ice for 30 minutes. The protein obtained from the supernatant after centrifugation at 13,000 rpm at 4°C for 10 minutes was quantified by the method of Bradford using a spectrophotometer (UV-1601 PC, Shimadzu, Japan) at 595 nm.

Poly (ADP-ribose) polymerase (PARP). Fifty mg of protein denatured on 9% SDS-polyacrylamide gel (30% acrylamide, 1.5 M Tris, 10% SDS, 10% APS, TEMED, 0.5M Tris) were electrophoresed and then transferred to a nitrocellulose membrane using a Hoefer transfer kit (Hoefer Scientific Instruments, San Francisco, CA). The nitrocellulose membrane was blocked with 5% non-fat dry milk/TNE-T buffer (10 mM Tris-Cl, 0.1 M NaCl, 1 mM EDTA, 0.05% Tween 20) and reacted with anti-PARP antibody (rabbit, polyclonal, Upstate Biotechnology, Lake Placid, NY) for 3 hours at room temperature. Anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) was reacted for 90 minutes. The band was verified using ECL hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK) and a developer (Fuji Photo Film, Fuji, Kanagawa, Japan).

Bcl-2 family. In order to determine the changes in the Bcl-2 expression, the protein (50 ng) denatured on 12% SDS-

polyacrylamide gel was electrophoresed. Anti-mouse Bcl-2 antibody (mouse, monoclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody and anti-mouse IgG-horseradish peroxidase (goat, Santa Cruz Biotechnology, Santa Cruz, CA) as the secondary antibody. For Bax, anti-Bax antibody (rabbit, polyclonal,

Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit IgG-horseradish peroxidase were used as the primary and secondary antibodies, respectively.

Statistical analysis

Statistical analysis was performed with the SPSSWIN statistical package program. Comparisons between the con-

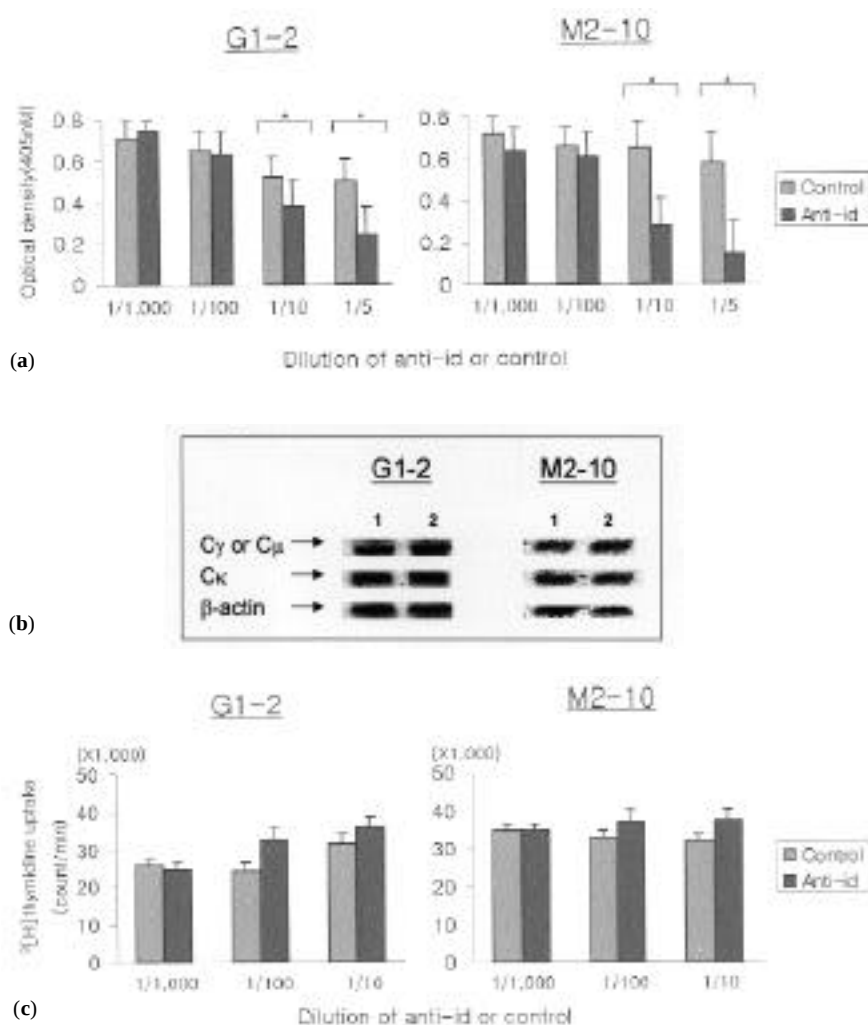


Fig. 1. The effect of anti-idiotypic antibody (anti-Id) on anti-dsDNA antibody production, transcription, and cellular proliferation of anti-dsDNA antibody producing cells. **(a)** To compare the titer of anti-dsDNA antibody (ab), ELISA was performed with culture supernatant after incubation with anti-Id or normal rabbit immunoglobulin (Ig) as control. Asterisks (*) denote a statistically significant difference ($p < 0.05$) compared with the control. The horizontal axis shows the amounts of dilution of anti-Id. The vertical axis shows the optical density that represents the amount of ab production.

In G1-2 hybridoma cells, at the dilutions of 10- and 5-fold ab production significantly decreased compared to that of the control. In M2-10 cells, the amount of ab production was also decreased by anti-Id. **(b)** Northern blot of Ig heavy chains (C γ C μ) and light chain (C κ) was performed to compare the transcription rate of Ig. β -actin used as an internal control. Anti-Id or control was diluted 10-fold. Lanes 1 and 2 represent control and anti-Id, respectively. Relative band densities of C κ C γ or C μ to β -actin were not significantly different between the control and anti-Id for both cells. **(c)** After G1-2 and M2-10 cells were cultured with control or anti-Id, [³H]thymidine was added and radioactivity was measured to compare cellular proliferation. [³H]thymidine uptake counts did not decrease after anti-Id treatment in both cells.

trol and anti-Id groups, between anti-Id alone and the combined treatment groups, and between prednisolone alone and the combined treatment groups were analyzed using the Wilcoxon matched-pairs signed-ranks test. P values less than 0.05 were considered statistically significant.

Results

Effects of anti-Id on antibody production, transcription and proliferation of anti-dsDNA antibody producing cells

In order to determine the best conditions for G1-2 and M2-10 cells to produce anti-dsDNA antibody, ELISA was performed on various concentrations of cells. The results, repeated 6 or 7 times, showed that the amount of antibody production was highest on day 2 at each cell concentration. Cell viability at the concentration of 5×10^4 cells/ml on the second day of culture was 69–82% and was 67–75% at 1×10^4 cells/ml. Thus, anti-Id and the control were cultured for 48 hours at 5×10^4 cells/ml.

The amount of anti-dsDNA antibody production was compared using ELISA. The concentration of anti-Id we prepared was 2–3 mg/ml, then diluted 1,000 fold, 100 fold, 10 fold and 5 fold. When anti-Id was added to G1-2 and M2-10 cells at 1: 1,000 and 1: 100, the amount of anti-dsDNA antibody production was not different from the control. When anti-Id was diluted by 10- and 5-fold, the amount of anti-dsDNA antibody production of anti-Id treated cells decreased significantly compared to the control (Fig. 1a). Thus, at concentrations higher than 0.2–0.3 mg/ml, anti-Id resulted in decreased anti-dsDNA antibody production. We conducted further experiments at concentrations higher than 0.2–0.3 mg/ml. To determine the effect of anti-Id on the transcription rate of the Ig gene of G1-2 and M2-10 cells, a northern blot was performed. The intensity of the bands on the Ig heavy and light chains did not show a significant difference (Fig. 1b). We tested ^3H thymidine incorporation to determine the effect of anti-Id on the proliferation of cells. The uptake of ^3H thymidine after 48 hours of culture

did not show any difference according to the concentration of treated anti-Id, and there was no difference between anti-Id treated and the control samples (Fig. 1c).

Effects of anti-Id on apoptosis of anti-dsDNA antibody producing cells

We hypothesized that the decrease of anti-dsDNA antibody production of G1-2 and M2-10 cells after anti-Id treatment was due to inducing apoptosis of these cells. The amount of apoptosis was evaluated by FACS analysis using annexin V-FITC and propidium iodide. When the G1-2 and M2-10 cells were treated with anti-Id or control, the number of cells that were stained with annexin V and propidium iodide was increased according to the duration of treatment in both anti-Id and control samples. After treatment of 8 hours, the apoptosis of anti-Id treated cells was more increased than the controls (Fig. 2a).

To confirm that apoptosis was induced by anti-Id, the DNA from G1-2 and M2-10 cells treated with anti-Id were electrophoresed. DNA laddering was verified in G1-2 cells at 7 hours after culture and in M2-10 cells at 5 hours (Fig. 2b).

We also checked caspase activation by measuring the cleavage of PARP. When caspases are activated by apoptotic signals, PARP is cleaved from a 116 kd whole molecule to an 85 kd fragment. The PARP cleavage was shown in G1-2 and M2-10 cells after treatment with anti-Id; lightening of the 116 kd band and darkening of the 85 kd band were verified (Fig. 2c).

The mechanism of apoptosis-induced by anti-Id treatment

For G1-2 and M2-10 cells, the expression of Fas and FasL was verified. To determine whether expressed Fas plays a role in apoptosis, these cells were treated with IgG anti-Fas antibody (Jo-2), which is known to induce apoptosis by binding to Fas. The viability of G1-2 cells decreased in proportion to the Jo-2 concentration, but the decrease in the viability of the M2-10 cells did not correlate with the Jo-2 concentration (data not shown). To determine the cor-

relation between FasL and apoptosis induced by anti-Id, G1-2 and M2-10 cells were treated with anti-Id and the degree of FasL expression was compared to that of the control. There was no difference between the control and anti-Id treated groups (data not shown). There is a report that the Bcl-2 family is involved in B cell receptor (BCR) ligation-induced apoptosis (25). Since it has been reported that the levels of Bcl-2 mRNA do not correlate well with the levels of Bcl-2 protein (26), we checked the levels of Bcl-2 and Bax protein to investigate the possible role of the Bcl-2 family in the apoptosis induced by anti-Id. When treated with anti-Id, the intensity of Bcl-2 gradually decreased with time in G1-2 and M2-10 cells; however, the intensity of the Bax band did not show any differences (Fig. 3).

It has been suggested that apoptosis may be caused by the accumulation of oxygen-free radicals (27). We evaluated cell viability after treatment with anti-oxidant (N-acetylcysteine, 1 mM). When G1-2 and M2-10 cells were treated with both N-acetyl-L-cysteine and anti-Id, the cell viability increased compared to those cells treated with anti-Id alone (G1-2; 79.1 ± 1.0 vs. $62.6 \pm 2.4\%$, $p < 0.05$, M2-10; 60.2 ± 5.1 vs. $50.1 \pm 5.5\%$, $p < 0.05$, at 4 hours), and the cell viability increased proportionally to the concentration of N-acetyl-L-cysteine (Fig. 4).

It has been reported that the activation of protein kinase C (PKC) and increase in $[\text{Ca}^{2+}]$ override the BCR-mediated induction of apoptosis (11). When G1-2 and M2-10 cells were treated with anti-Id and calcium ionophore A23187 (50 nM) and phorbol 12-myristate 13-acetate (10 nM), which are known to participate in many stages of cell survival, the cell viability of G1-2 cells did not show any difference, whereas that of M2-10 cells showed higher than anti-Id alone (41.5 ± 6.2 vs. $54.5 \pm 7.9\%$, $p < 0.05$, at 22 hours; Table I).

The effect of CS on apoptosis of anti-dsDNA antibody producing cells

CS is commonly used to treat lupus nephritis and is known to induce lymphocyte apoptosis. The induction of apop-

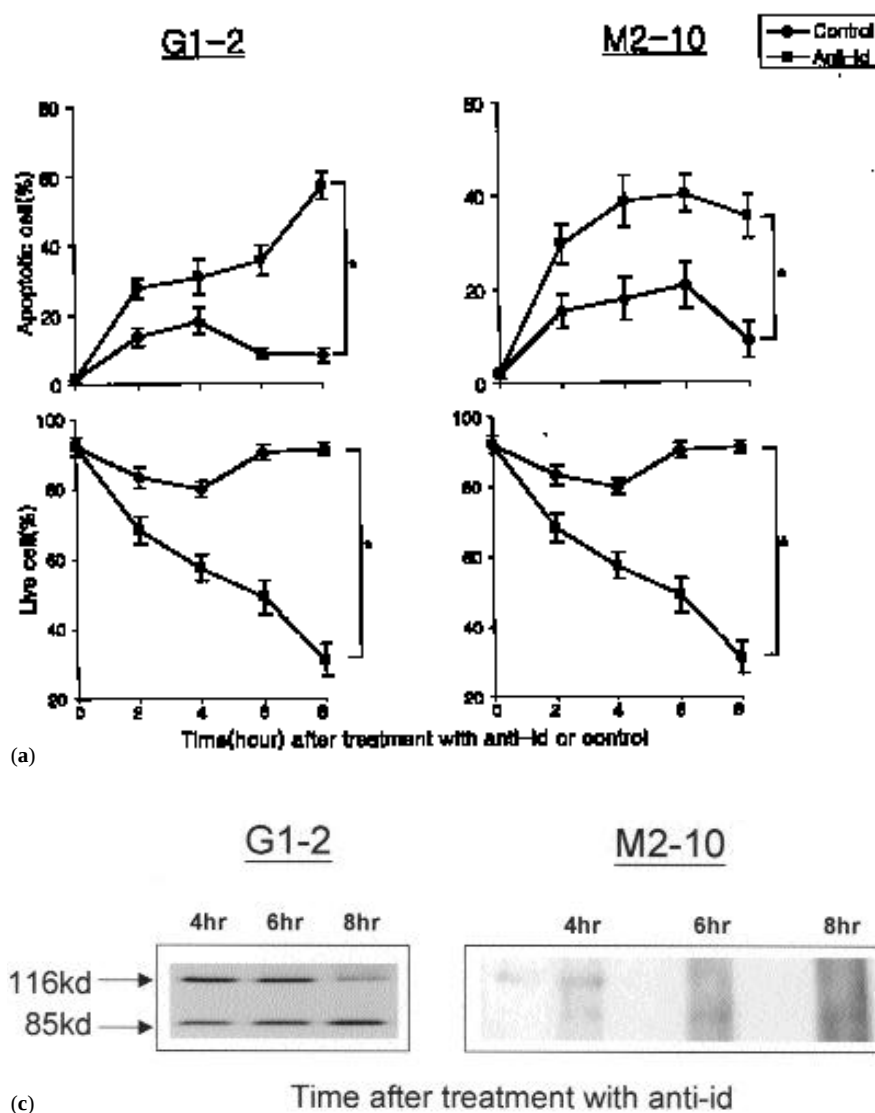


Fig. 2. The effect of anti-idiotypic antibody (anti-Id) on apoptosis in anti-dsDNA antibody producing cells. **(a)** Cell viability and apoptosis of anti-Id treated G1-2 and M2-10 cells. After incubation with anti-Id and control, cells were stained with annexin V-FITC and propidium iodide. The percentage of apoptosis in anti-Id treated cells significantly increased compared to the control. Asterisks (*) denote a statistically significant difference ($p < 0.05$) compared with the control. **(b)** DNA electrophoresis after anti-Id treatment in G1-2 and M2-10 cells. After 5 hours (M2-10) and 7 hours (G1-2) of incubation, electrophoresis was performed. Lanes 1 and 2 represent the control and anti-Id, respectively. DNA laddering was found in the anti-Id treatment group. M: DNA molecular size marker; **(c)** Caspase activation evaluated by the cleavage of poly (ADP-ribose) polymerase (PARP). G1-2 and M2-10 cells were incubated with anti-Id and analyzed by Western blot using anti-PARP antibody. After anti-Id treatment, the 85 kd band got darker, but the 116 kd band became lighter in G1-2 and M2-10 cells.

tosis of G1-2 and M2-10 cells by CS was verified. The classic DNA laddering pattern was observed after treat-

ment with CS on both G1-2 and M2-10 cells (Fig. 5a). When treated with CS, the cell viability decreased compared

to that of the control and this decrease was proportional to the concentration of CS (Fig. 5b). We determined the prednisolone concentration (G1-2: 50 nM, M2-10: 10 nM) where the cell viability could be maintained between 50% – 60% and next experiment was conducted. The degree of FasL expression in G1-2 and M2-10 cells after treatment with CS did not show any difference compared to that of the control (data not shown). When treated with both N-acetyl-L-cysteine (1 mM) and CS, the cell viability increased compared to CS only (Fig. 5c). By adding calcium ionophore A23187 and phorbol 12-myristate 13-acetate, the cell viability of M2-10 cells increased compared to CS only (Table II), but G1-2 was not.

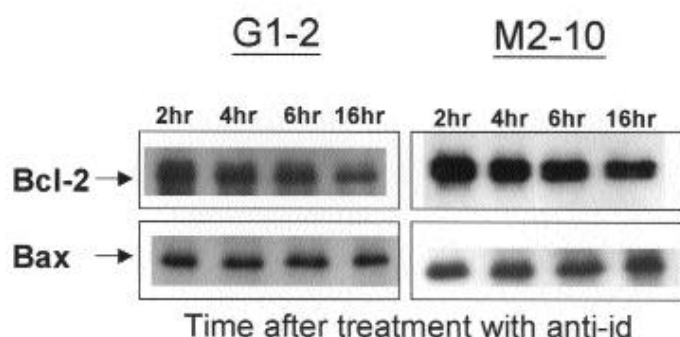


Fig. 3. Western blot of Bcl-2 and Bax after anti-idiotypic antibody (anti-Id) treatment in anti-dsDNA antibody producing cells. G1-2 and M2-10 cells were treated with anti-Id and analyzed using anti-Bcl-2 antibody and anti-Bax antibody. Bcl-2 expression decreased with time, but Bax expression did not change.

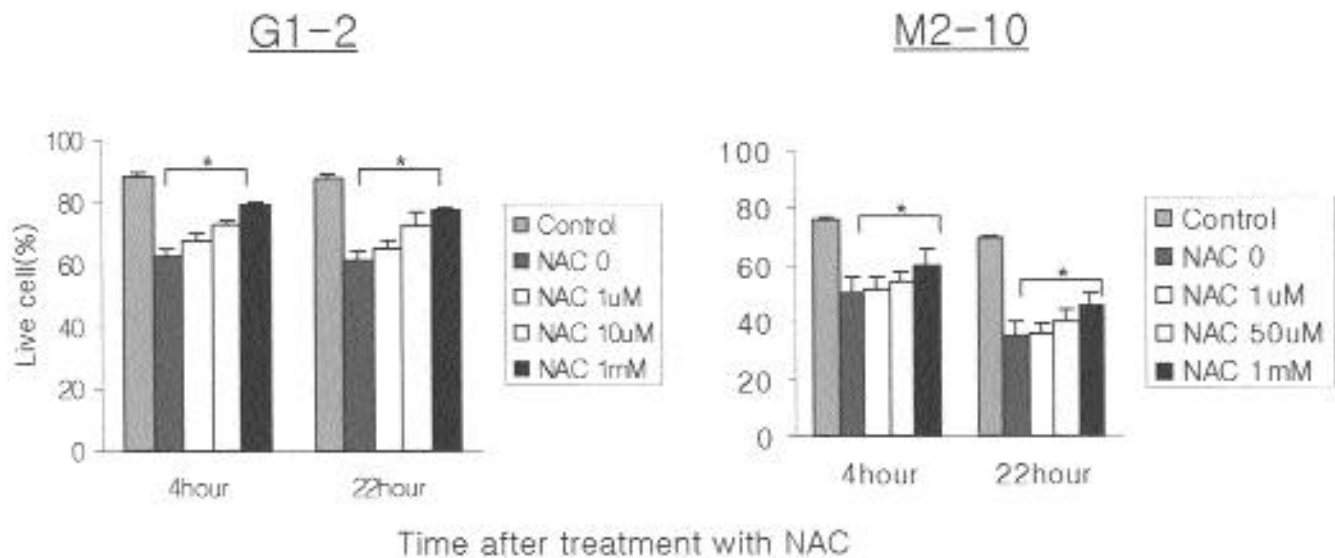


Fig. 4. Changes in the cell viability after adding N-acetyl-L- cysteine (NAC) to G1-2 and M2-10 cells treated with anti-idiotypic antibody (anti-Id). After anti-Id treatment with or without NAC, both cells were stained with annexin V and propidium iodide. With the increase in the concentration of NAC, the number of live cells rose in both cells. Asterisks (*) denote a statistically significant difference ($p < 0.05$) compared with only anti-Id.

Table I. Change in the cell viability after treatment with anti-Id or anti-Id plus PMA with Ca in G1-2 and M2-10 cells.

		Cell viability (%)	
		Anti-Id	Anti-Id + PMA with Ca
G1-2	3 hours	77.5 $\pm$ 4.0	73.5 $\pm$ 2.6
	22 hours	73.8 $\pm$ 6.3	67.8 $\pm$ 2.8
M2-10	3 hours	64.5 $\pm$ 3.3	65.8 $\pm$ 2.0
	22 hours	41.5 $\pm$ 6.2	54.5 $\pm$ 7.9*

* denotes a statistically significant difference ($p < 0.05$) compared with anti-Id treatment.

Table II. Changes of cell viability after treatment with PDL or PDL plus PMA with Ca in G1-2 and M2-10 cells.

		Cell viability (%)	
		PDL	PDL + PMA with Ca
G1-2	3 hours	77.1 $\pm$ 2.1	70.7 $\pm$ 1.8
	22 hours	63.3 $\pm$ 5.5	59.0 $\pm$ 3.7
M2-10	3 hours	64.2 $\pm$ 3.5	65.7 $\pm$ 1.6
	22 hours	28.2 $\pm$ 6.6	41.3 $\pm$ 6.5*

*Denotes a statistically significant difference ($p < 0.05$) compared with the PDL treatment group.

Discussion

The spontaneous production of multiple antibodies to a variety of self-antigens is one of the characteristics of SLE. Among these, anti-dsDNA antibody is considered a hallmark of SLE and the titer of anti-dsDNA antibody also rises when the disease is active (1-3). These observations have led to the consideration that specific manipula-

tion of the production of this autoantibody might be one recommended approach to therapy. Since the idiotype-anti-Id network is believed to control the production of antibodies (28), SLE could be controlled by decreasing anti-dsDNA antibody production by administering anti-Id against anti-dsDNA antibody in animal models (4-9). In SLE patients who spontaneously re-

cover from autoimmune disease, anti-Id may play an important role (29). In fact, autologous anti-Id against autoantibody has been demonstrated in the serum of patients experiencing remission in SLE (30). Also, intravenous immunoglobulin (IVIG) has been reported to be effective in treating murine and human SLE (31,32). This may be mediated through manipulation of the idiotype network and the neutralization of pathogenic autoantibodies (31). The purpose of the present study was to elucidate the mechanism of decreased anti-dsDNA antibody production after anti-Id treatment in an *in vitro* model. We first performed the experiments to see if the anti-Id that we prepared would decrease anti-dsDNA antibody production. As expected, the production of anti-dsDNA antibody was decreased at concentrations higher than 0.2 – 0.3 mg/ml.

Possible mechanisms to be considered for the decrease in anti-dsDNA antibody due to anti-Id are: (1) a decrease in anti-dsDNA antibody produced per unit cell, or the unbalanced production of heavy and light chains, (2) a decrease in cell proliferation after anti-Id treatment, and (3) a decrease in the number of cells after anti-Id treatment. To address the first possibility, we performed northern blots on anti-dsDNA antibody producing cells after anti-Id

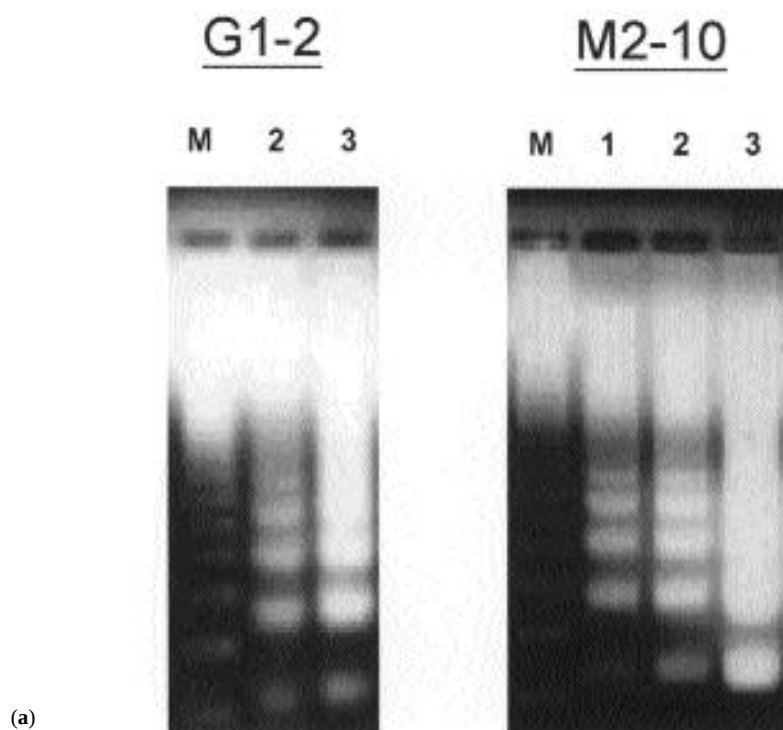
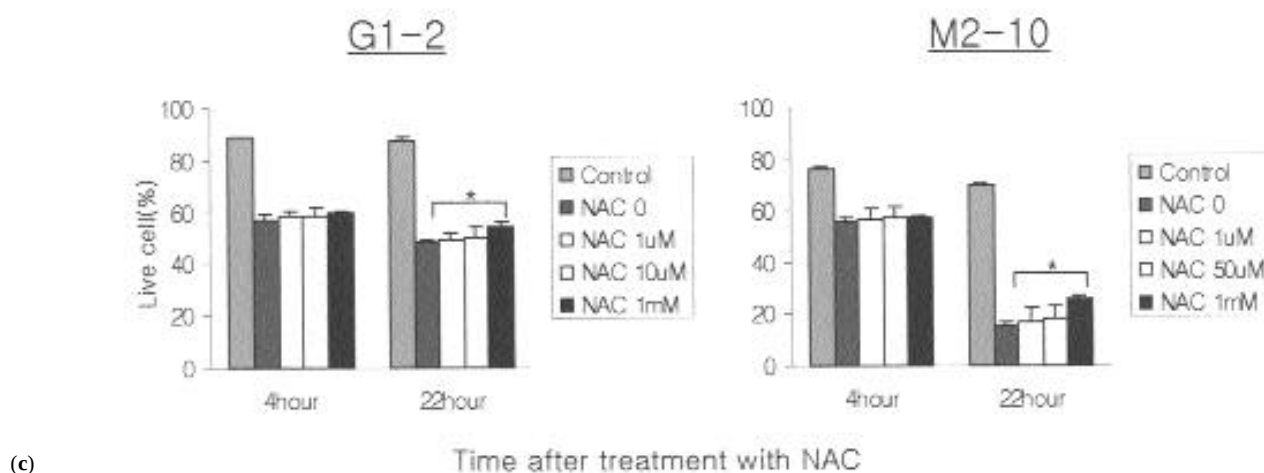
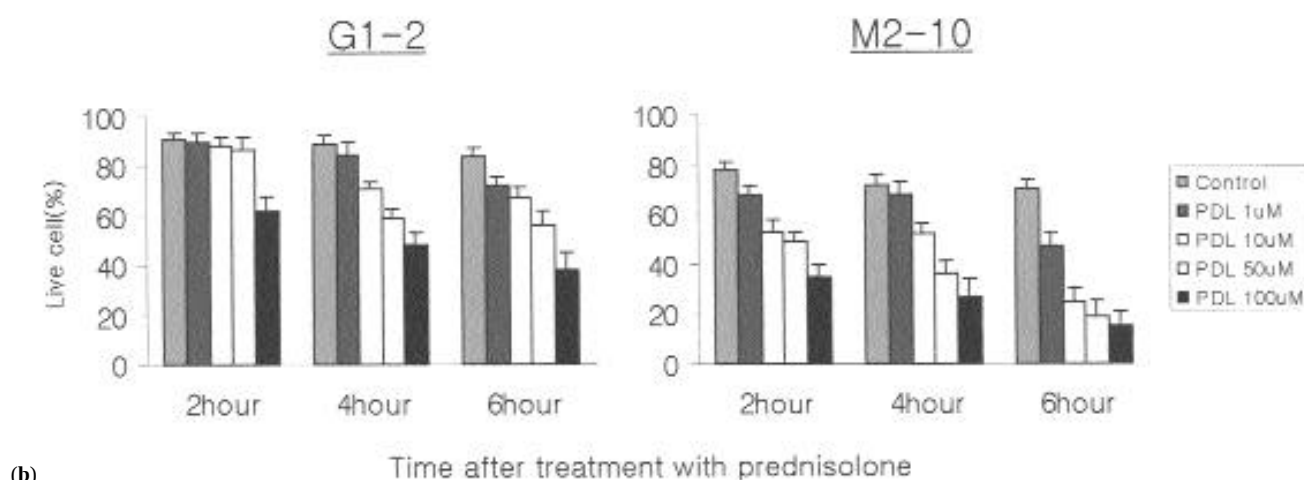


Fig. 5. The effect of corticosteroids on apoptosis in anti-dsDNA antibody producing cells. **(a)** DNA electrophoresis after prednisolone (PDL) treatment in G1-2 and M2-10 cells. After 10 hours (M2-10) and 12 hours (G1-2) of incubation, electrophoresis was performed. Lanes 1, 2 and 3 represent 1, 10 and 50 nM of PDL, respectively. DNA laddering was observed in both cell lines. **M:** DNA molecular size marker. **(b)** Change of cell viability after PDL treatment in G1-2 and M2-10 cells. Both cells were incubated with PDL and stained with annexin V and propidium iodide. The percentage of live cells decreased according to the concentration of PDL. Asterisks (*) denote a statistically significant difference ($p < 0.05$) compared with the control. **(c)** Change of the cell viability after adding N-acetyl-L-cysteine (NAC) treatment in G1-2 and M2-10 cells treated with corticosteroids. After prednisolone (PDL) treatment with or without NAC, both G1-2 and M2-10 cells were stained with annexin V and propidium iodide. Concentration of PDL in G1-2 was 50 nM, and M2-10 was 10 nM. At 22 hours treatment, NAC increased the number of live cells in both cells. Asterisks (*) denote a statistically significant difference ($p < 0.05$) compared with PDL treatment alone.



treatment and showed that there was no decrease in the transcription of Ig genes, and that the bands for the heavy and light chains also did not show any difference. To approach the second mechanism, we showed that cell proliferation did not decrease, even at concentrations where anti-dsDNA antibody production was suppressed. When the cells were treated with anti-Id, however, the levels of cell death increased. In order to find out whether this cell death was due to simple necrosis or apoptosis, we checked the percentages of living cells and apoptotic cells at the same time. The results showed that living cells initially decreased drastically after anti-Id treatment, and that apoptotic cells drastically increased in proportion to this decrease. Also, by demonstrating a ladder pattern on DNA electrophoresis and the cleavage of PARP, we confirmed that cell death was due to apoptosis. Thus, the decrease in anti-dsDNA antibody after treatment with anti-Id was due to the apoptosis of anti-dsDNA antibody producing cells. This result is similar to those of other studies where apoptosis was induced from BCR ligation with anti-Ig antibody (10, 11, 33).

The MRL-*lpr* mouse, which has a mutation in the Fas gene, spontaneously produces several autoantibodies including anti-dsDNA antibody (20, 21). This mouse develops nephritis about 20 weeks after birth, coincident with the production of anti-dsDNA antibodies. It seems likely that there is a causal relationship between imperfect Fas-mediated apoptosis and the development of murine SLE (34). We obtained two anti-dsDNA producing hybridomas from one of these mice. Thus, we needed to determine whether the cells used in this study would express Fas and FasL. While the cells used in this study expressed both Fas and FasL, the G1-2 cells responded to agonistic anti-Fas antibody, while the M2-10 cells did not. There was no difference in FasL expression between anti-Id-treated and control hybridoma cells. It is probable that the apoptosis of these cells due to anti-Id treatment was not related to the Fas-FasL pathway. Transgenic mice overex-

pressing Bcl-2 (35) develop prolonged B-cell survival and other features in common with SLE, suggesting that this molecule, which plays a crucial role in apoptosis (14), may play a role in the pathogenesis of SLE. Bcl-2 family protein expression was also evaluated; Bcl-2 expression of G1-2 and M2-10 decreased with time after anti-Id treatment, but there was no difference in Bax expression. There is a report that increased Bcl-2 blocks apoptosis in several lymphoid cell lines, but this is the first report that anti-Id treatment decreases Bcl-2 protein levels. It is of interest that these cells were rescued from anti-Id induced apoptosis by agents that activate PKC in conjunction with an increase in $[Ca^{+2}]$. Thus, the signaling pathway in these cells appears not to involve the classical phosphatidylinositol 4,5-bisphosphate hydrolysis pathway that results in the activation of PKC along with an increase in $[Ca^{+2}]$. Anti-Id induced apoptosis was also inhibited with antioxidant. These results suggest that Bcl-2, oxygen-free radicals and the PKC pathway may participate in anti-Id induced apoptosis.

In addition, the effects of CS, the basic drug for treating SLE, on anti-dsDNA antibody production cells were evaluated. It is known that CS may induce apoptosis of lymphoid cells (18, 19). Compelling evidence indicates that DNA binding and the subsequent transcriptional regulation of specific genes is required for this process. However, the exact mechanism by which apoptosis occurs in lymphocytes is unknown. We verified the presence of dose-dependent CS-induced apoptosis in anti-dsDNA antibody producing cells. The apoptosis induced by CS was not dependent on the Fas-FasL pathway; similar to anti-Id induced apoptosis, Bcl-2, oxygen-free radicals and the PKC pathway were involved.

In summary, the decrease in anti-dsDNA antibody production of anti-dsDNA producing cells by anti-Id is due to the induction of apoptosis. The mechanism of anti-Id induced apoptosis shares at least some molecular pathways with CS-induced apoptosis. Considering the adverse effects of CS, anti-Id treatment is a reasonable alternative.

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