行政院國家科學委員會專題研究計畫 成果報告

抗去氧核醣核酸酵素抗體在全身性紅斑狼瘡之角色

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中文摘要

抗去氧核酸酵素 (DNase) 是分解 DNA 的主要分子，其功能異常可能導致在全身性紅斑狼瘡病人抗 DNA 抗體的產生。我們以牛 DNaseI 固定在 ELISA 盤篩檢在 SLE 病人血清中抗 DNase 抗體的含量發現有 62% 病人呈現陽性，正常人則只有 8% 呈現陽性抗 DNase 抗體。抗 DNase 抗體和抗 DNA 抗體也呈正相關。用親合性分離法從 SLE 病人血清中純化之抗 DNase IgG 抗體可以同時和 DNase 及 DNA 結合而且可以完全抑制 DNase 的作用，用 DNase 免疫 NZB/NZW 小鼠可使之產生抗 DNase 及抗 DNA 抗體並加速蛋白尿的產生，因此抗 DNase 抗體可能在 SLE 致病機轉中伴演促進的角色。本結果已發表在 J. Biomedical Science 10: 544-551, 2003。

關鍵字: 自體抗體、自體免疫、交又反應
Abstract

Deoxyribonucleases (DNases) are key enzymes for digesting DNA. Abnormalities in the function of these enzymes may contribute to the development of anti-DNA antibodies in systemic lupus erythematosus (SLE). In this study, we used bovine DNase 1-coated ELISA plates to screen anti-DNase antibodies in SLE patients. About 62% of the sera of SLE patients (63/101) were positive for anti-DNase antibodies, compared to only 8% of normal controls (8/98). A positive correlation was also found between the concentrations of anti-DNase and anti-DNA antibodies in sera of SLE patients. Affinity-purified anti-DNase immunoglobulin G (IgG) from pooled sera of SLE patients bound to bovine DNase as well as DNA. A synthetic peptide, corresponding to the catalytic site of DNase, was able to completely inhibit the binding of anti-DNase IgG to DNase. In addition to bovine DNase, the anti-DNase IgG also bound to and inhibited the enzymatic activities of DNase present in streptococcal supernatants and human urine. Immunization of lupus-prone NZB/NZW mice with bovine DNase enhanced the production of anti-DNase and DNA antibodies, and accelerated the occurrence of proteinuria. Taken together, these results suggest that DNase-inhibitory antibodies which recognize a conserved epitope near the catalytic site of DNase may act in the pathogenesis of SLE. (Published on J. Biomedical Science 10: 544-551, 2003)

Key Words  Autoantibodies; Autoimmunity; Cross-reactivity; DNA
**Introduction**

Systemic lupus erythematosus (SLE) is a prototypical systemic autoimmune disease, which is characterized by the presence of anti-nuclear antibodies (ANA) directed against DNA and nucleosomes. The resulting immune complexes are deposited in blood vessels and filter organs such as joints and the kidney, causing vasculitis, arthritis, and glomerulonephritis. The etiology of SLE is unknown, but several studies suggest that inadequate clearance of potential autoantigen, such as nuclear DNA-protein complexes after cell death may contribute to the loss of self-tolerance in SLE (21).

Deoxyribonuclease (DNase), which occurs in almost all living organisms, is a group of enzymes capable of hydrolyzing DNA, and at least two genes, DNase I and II, are known. Bovine pancreatic DNase I was the first discovered and is the best characterized DNase (6, 11). It consists of a single polypeptide of 260 amino acids and two disulfide bridges. The crystal structure of bovine DNase 1, complexed to a short oligonucleotide has been solved. An exposed loop of DNase I (amino acids 73-78, Arg-Asn-Ser-Tyr-Lys-Glu) binds in the minor groove of DNA, probably through electrostatic interactions (10, 16, 17). In addition, the active site of DNase is phylogenetically highly conserved among DNase from different species. As in cows, human DNase also exhibits polymorphism and can be
detected in different tissues such as sera, urine, kidney, liver, and pancreas (4, 6).

Previous studies have shown that there are low serum and urine DNase activity in both
SLE patients and SLE-prone NZB-NZW mice (2, 8). Moreover, DNase 1-deficient mice
generated by gene targeting show the classical symptoms of SLE, which are the presence
of ANA and glomerulonephritis (9). Mutation of DNase 1 gene has also been found in
some SLE patients (23), although, not all SLE patients show defects in DNase genes (1,
15, 19). Therefore, multiple factors are probably involved in causing the decrease of
serum DNase activity in SLE. In this study, we examine the prevalence of anti-DNase
antibodies in SLE patients and use an affinity column to purify anti-DNase antibodies from
SLE patients’ sera to analyze their antigenic specificity and their effects on DNase
enzymatic activity. Finally, DNase was immunized in NZB/NZW mice to study the
pathogenic effects of anti-DNase antibodies in the disease development of SLE. Results
from these studies indicate anti-DNase antibodies in SLE may interfere with DNase
activity and accelerate the disease process.
Materials and Methods

*Human sera*

Serum samples were collected from 101 SLE patients fulfilling at least four of the revised 1982 criteria for SLE (18) and stored at $-70^\circ\text{C}$. There were 85 females and 16 males, age range 4-64 years with mean of 30 years. In addition, 98 normal serum samples without antinuclear antibodies (ANA) were used as controls.

*Bovine DNase I*

Bovine DNase I was purchased from Sigma Chemical Company (St. Louis, MO). SDS-PAGE and zymograph confirmed the purity and activity of this enzyme, respectively. There was confirmed to be no DNA contamination in the bovine DNase I preparation because bovine DNase I solution (1 mg/ml) showed no absorption at 260 nm and ethidium bromide staining of this DNase solution run on agarose gel was also negative (data not shown).

*Synthetic peptides*

Synthetic peptide (DNase peptide) corresponding to the catalytic domain of DNase (amino acids 73-78) (12) was synthesized by the Peptide Synthesis Center, National
Science Council, Taipei, Taiwan. The sequence of this peptide is Ala-Arg-Asn-Ser-Tyr-Lys-Glu-Ala. An additional peptide (Glu-Leu-Lys-Cyc-Tyr-Thr-Cyc-Lys-Glu) was used as a negative control. The purity of these peptides was confirmed by HPLC and amino acid analysis.

*Mice immunization*

Five to six-week-old female NZB/NZW mice were used in this study. These mice were originally purchased from Jackson Laboratory (Bar Harbor, ME) and bred in the Laboratory Animal Center, National Cheng Kung University. DNase (50 μg/mouse) in complete Freund’s adjuvant was intraperitoneally injected into 6-month-old NZB/NZW mice. At two and four weeks, these mice were boosted in the same route as primary immunization with DNase 1 in incomplete Freund’s adjuvant. Sera were collected from the axial plexus of the mice at times as indicated. In addition, urine was collected from each group of mice at ages as indicated. The protein level in the urine was measured by BCA protein assay (Pierce; Rockford, IL).

*Purification of immunoglobulin by protein A and DNase affinity column*
IgG from normal sera was purified using a protein A affinity column (Pharmacia Biotech; Piscataway, NJ). Unbound components were washed away with 0.85% saline. Bound IgG was then eluted with 0.1 M glycine (pH 3.0), and the pH of the eluent was neutralized to 7.0 with 1 M Tris (pH 8.0). For purification of anti-DNase antibodies, sera from SLE patients with high anti-DNase Ab titer were pooled and passed through a DNase affinity column followed by a protein A column. Briefly, bovine DNase I was conjugated to Sepharose 4B (Pharmacia Biotech) according to the manufacturer’s procedures. Pooled sera were passed through the DNase affinity column and eluted with 3 M MgCl₂. The eluted proteins were dialyzed against PBS and anti-DNase IgG was further purified by protein A column. The concentration of immunoglobulin was adjusted by ultrafiltration (Amicon; Beverly, MA) and determined by BCA protein assay.

**Enzyme-linked immunosorbent assay (ELISA)**

Ninety-six well flat-bottom ELISA plates (Nunc, Denmark) were coated with 100 µl of either bovine DNase I (0.05 mg/ml in PBS) or synthetic peptides for two hours at 37 °C. After washing with PBS, the plates were blocked with a blocking buffer (1% bovine serum albumin in PBS) for 1 h at 37 °C. Then either 100 µl of diluted serum (100-fold in blocking buffer) or affinity-purified anti-DNase IgG was added and incubated for 2 h at 37 °C. Bound antibodies were detected by horseradish peroxidase-conjugated goat anti-human Ig or
anti-mouse Ig antibodies (Sigma) followed by OPD substrate (Sigma). ELISA plates were read by a Vmax microplate reader (Molecular Device, Menlo Park, CA) at 490 nm. To detect DNA cross-reactive antibodies, plates were first pre-coated with 100 µl of methylated BSA (10 µg/ml in 1N acetic acid and PBS) for 2 h at 37 ℃ and then coated with 100 µl of calf thymus deoxyribonucleic acid (Sigma; 2.6 µg/ml in PBS). In competitive inhibition assays, anti-DNase IgG (0.5 mg/ml) was pre-incubated with different amounts of DNase peptide or control peptide for 1 h at 37 ℃ before being added to the DNase or DNA-coated ELISA plates.

Collection of supernatants of *Streptococcus pyogenes* and human urine

Two different local isolates of *Streptococcus pyogenes* were grown in tryptic soybroth with yeast extract medium at 37 ℃ for 24 h. Supernatants of the culture medium were collected after centrifugation at 2500 g for 10 min. In addition, human urine was also collected from normal persons. The concentrations of both culture supernatants and human urine were adjusted by ultrafiltration (Amicon) before use.

SDS-PAGE and Western blot analysis

Bovine DNase I (1 mg/ml), human urine (4 mg/ml), and the supernatants of streptococcal culture (3 mg/ml) were separated by 12% SDS-PAGE with Laemmli’s
discontinuous buffer system (5). Electrophoresis was conducted for 1 h at 100 V and the proteins were detected by staining with Coomassie blue solution. In Western blot, proteins were transferred from SDS-PAGE to nitrocellulose sheets using the same procedure as previously described by Towbin (20). DNase was detected using anti-DNase IgG (100 µg/ml), horseradish peroxidase-conjugated anti-human immunoglobulin antibodies (Sigma), and substrate diaminobenzidine (Sigma).

**Zymographic assay for DNase**

The DNase activity in human urine and supernatants of streptococcal cultures was determined by zymographic assay (13). The samples were mixed with 2ME-free loading buffer and heated at 100 ºC for 3 min before being separated by SDS-PAGE in a polyacrylamide gel that contained 25 µg/ml of thymus DNA. Following electrophoresis, the gel was rinsed with water and placed in 50 ml buffer containing of 0.04 M Tris-HCl, 2 mM MgCl₂, and 0.02% sodium azide, pH 7.5. After being shaken gently at room temperature for 2 h, it was rinsed again with water and incubated in the same buffer overnight at 4 ºC. The gel was transferred to fresh buffer and further incubated for 2 h at room temperature. Ethidium bromide (final concentration, 1 µg/ml) was added to the solution for 30 min before viewing under UV light. DNase activities appeared as dark bands on a fluorescent background.
**DNase activity assay**

The catalytic activity of DNase in different samples was assayed using a method as previously described (3). Briefly, 0.1 ml sample solution was added to a mixture consisting of 0.1 ml of 0.4 % thymus DNA in 0.05M Tris Ca\(^{++}\)/Mg\(^{++}\) buffer containing 0.05 M MgCl\(_2\) and 0.05 M CaCl\(_2\), pH 7.2. After incubation at 37\(^\circ\)C for 1 h, the reaction was stopped by addition of 0.2 ml of 0.1 N HCl, followed by 0.8 ml of absolute ethanol. After standing for 5 min at room temperature, the mixture was centrifuged at 1,530 g for 10 min and the absorbance of the supernatant at 260 nm was measured. One unit of DNase activity was defined as the amount of enzyme that required to increase the absorbance of the supernatant at 260 nm by 1.0 in 10 min under the above conditions. In inhibition assays, different samples (about 0.03-0.04 unit) were incubated with 0.3 mg/ml of anti-DNase IgG or normal IgG in 0.1 ml of 0.05 M Tris-Ca\(^{++}\)/Mg\(^{++}\) buffer at 37\(^\circ\)C for 1 h, the remaining activities were then measured as described above.

**Statistical analysis**

Statistical analysis of data was performed using Student’s *t*-test, and differences were considered significant if *P* values were ≤0.05. Furthermore, sera with absorbance above the mean + 2 standard deviation (SD) of the normal human sera were considered positive.
Results

Presence of anti-DNase antibodies in SLE patients

There were 63 out of 101 of the SLE patients’ sera (positive rate 62.4%) which had a mean anti-DNase antibody by levels greater than the cut-off value (mean+2SD of the normal sera, 0.287), while only 8 out of 98 normal sera (8%) were positive for anti-DNase (fig. 1). The anti-DNase antibody levels in SLE sera were also significantly higher than those in normal sera (0.407±0.229 vs. 0.101±0.093, P<0.005) (fig. 1). Furthermore, using anti-human IgG specific antibodies as the secondary antibody, we noticed that most of these anti-DNase antibodies belonged to IgG isotype (data not shown). A positive correlation was also demonstrated between the anti-DNase antibody levels and anti-DNA antibody levels in SLE patients’ sera (with r= 0.727) (fig. 2).

Affinity purified anti-DNase IgG bound to DNase peptide as well as DNA

Figure 3 shows that anti-DNase IgG bound to matrix-bound DNase, DNase peptide and DNA, but not to control peptide, in an almost identical dose-dependent manner. Furthermore, the binding of anti-DNase IgG to DNase or DNA was inhibited by the DNase peptide but not by the control peptide (fig. 4). However, DNase peptide was more effective at inhibiting anti-DNase IgG binding to DNase than to DNA. DNase peptide at 0.5 mg/ml
caused 93% inhibition of anti-DNase IgG binding to DNase but only 32% to DNA.

**Anti-DNase IgG bound to bovine, human, and streptococcal DNase**

To confirm DNase activity, purity and immunogenicity, zymograph, SDS-PAGE and Western blot analysis were performed. Bovine DNase I showed a major band at about 34 KD in the SDS-PAGE, zymograph and Western blot (fig. 5, lane 2). When calf thymus DNA was loaded, no band was detectable in SDS-PAGE, zymograph and Western blot (data not shown). In addition, when human urine was loaded (fig. 5, lane 3), several bands were detected in SDS-PAGE but only two major and one minor bands were detected in Western blot. In zymograph, urine sample showed only these two major and one minor bands with DNase activity. Because in zymograph, the samples were run in non-reduced condition, the locations of bands were different from that in SDS-PAGE. In the supernatants of streptococcus only one band was recognized in SDS-PAGE, zymograph and Western blot (fig. 5, lanes 4 and 5).

**Anti-DNase IgG inhibited the enzymatic activities of bovine, human, and streptococcal DNase**

To further verify that anti-DNase IgG indeed can recognize DNase and inhibit its enzymatic activity, an inhibition assay was performed, in which pre-incubation of
anti-DNase IgG with bovine DNase caused a dose-dependent inhibition of the bovine DNase activity (fig. 6). Normal IgG also induced inhibition of bovine DNase after co-incubation; however its efficiency was much less than that of anti-DNase IgG (26% vs. 59%). This was probably due to non-specific inhibition or the presence of some anti-DNase antibodies in normal sera (fig. 1). Similar inhibitory effects were observed on human urine and streptococcal supernatants (fig. 6). Again, results showed that anti-DNase IgG was 2 times more potent than normal IgG at inhibiting DNase activities in human urine and streptococcal supernatants.

**DNase immunization in NZB/NZW mice enhanced the production of both anti-DNA and anti-DNase antibodies production and accelerated the occurrence of proteinuria**

To understand the pathogenic roles of anti-DNase in the disease development of SLE, DNase was immunized in lupus-prone NZB/NZW mice. In naïve mice, both anti-DNA and DNase antibodies in the sera were increased along with the ages, reflecting the lupus-prone nature of these mice. Immunization of NZB/NZW with DNase, however, significantly increased both anti-DNA and anti-DNase antibodies levels in the sera after boosting, as compared to those in control mice (fig. 7). In addition, the amounts of protein in urine of 8-month old DNase-immunized mice were also significantly increased, as compared to those in control mice (fig. 8). Nevertheless, at 10 months of age,
spontaneous development of SLE may also occur in control mice. Therefore, no significant difference of proteinuria was found in both DNase-immunized and control mice.
Discussion

DNase plays an important role in the clearance of apoptotic products, such as DNA-protein complexes. Inadequate clearance of these potential autoantigens may contribute to the loss of self-tolerance in SLE. Indeed, low serum activity of DNase has been found in SLE. Defective DNase gene as well as the presence of the DNase inhibitor, actin, have been suggested as being responsible for the decrease of serum DNase activity in SLE. In this study, we demonstrate that there was a high prevalence of anti-DNase antibodies which can inhibit DNase function in SLE patients. In addition, a good correlation between the concentrations of anti-DNase and anti-DNA antibodies in SLE patients was also found. Therefore, DNase-inhibitory antibodies may provide another way to interfere with DNase function in SLE.

To further characterize the relationship between anti-DNase and anti-DNA antibodies, we used DNase and protein A affinity column to purify anti-DNase IgG from SLE patients' sera. The affinity-purified anti-DNase IgG was able to bind to DNase and DNA which was inhibited by the presence of DNase peptide but not control peptide. Since no contamination of DNA was found in DNase solution and no DNase was detectable in the calf thymus DNA used in this study, these results indicate that there are cross-reactive epitopes shared by DNase and DNA. This suggestion is supported by a previous report.
which shows that some of the monoclonal anti-DNA antibodies are able to cross-react with bovine DNase I and inhibit its function (12). However, the precise mechanism of the cross-reactivity of anti-DNA and anti-DNase antibodies to each other’s ligand required further studied.

The origin of anti-DNase antibodies in SLE patients is unknown. There are at least three different possible mechanisms to induce anti-DNase antibodies in SLE. The first is that anti-DNase antibodies may be induced as a consequence of infection. This hypothesis is supported by the cross-reactivity of anti-DNase antibodies to streptococcal DNase. The second possibility is that anti-DNase antibodies might arise from an immune response to a complex of DNA and DNA binding proteins when there are excess apoptosis or defects in the clearance of dying cells (21). Last but not least, anti-DNase antibodies might be generated through anti-idiotypic network against anti-DNA antibodies. In this regard, DNA-hydrolyzing antibodies which mimic DNase may represent such an idiotypic cascade (14). These three possibilities are not mutual exclusive. Therefore, anti-DNase antibodies in SLE may be generated by different mechanisms.

The pathological role of anti-DNase antibodies in the disease development of SLE was demonstrated by the acceleration of proteinuria in DNase immunized NZB/NZW mice. Even though we did not test the DNase-inhibitory activities of these murine anti-DNase antibodies, previous study has shown that anti-DNase antibodies generated in a
DNase-immunized animal can inhibit DNase activity (7). Therefore, it is possible that anti-DNase antibodies in DNase-immunized NZB/NZW mice may inhibit DNA degradation, enhance anti-DNA antibody production and subsequently, accelerate proteinuria occurrence. However, DNase immunization in normal mice (BALB/c), which also induced high titers of anti-DNase and anti-DNA antibodies, did not cause significant pathological changes in these mice (data not shown). Therefore, anti-DNase antibodies are probably involved in disease progression but not in disease initiation. This may explain why not all SLE patients have high anti-DNase antibodies and anti-DNase antibodies were also found in some of the normal sera. Other factors such as genetic susceptibility or environmental factors which are important in the regulation of apoptosis and immune activation, may contribute to the development of SLE (22). In summary, the etiology of SLE is complex and multifactorial, and a better understanding of the mechanisms of disease is essential to design effective treatment.
Acknowledgments

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References


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Figure legends:

**Fig. 1.** Anti-DNase antibodies in SLE patients' and in normal sera. Anti-DNase antibodies were detected using bovine DNase I coated ELISA plates, as described in Materials and Methods. The levels of anti-DNase antibodies in normal sera (n=98) were compared with those of SLE patients (n=101). Horizontal lines denote mean anti-DNase antibody levels of SLE patients' and normal sera.

**Fig. 2.** Correlation of anti-DNase and anti-DNA antibodies in SLE patients' sera. Ten microliters of 100-fold diluted sera from SLE patients' sera (n=30) were incubated with DNase- or DNA-coated plates and detected by ELISA, as described in Materials and Methods. Data represent mean of triplicate.

**Fig. 3.** Affinity-purified anti-DNase IgG cross-reacts to DNase, DNase peptide, and DNA. Different concentrations of affinity-purified anti-DNase IgG were incubated with either DNase-, DNase peptide-, control peptide- or DNase-coated plates, as described in Materials and Methods. Data represent the mean ± SD of three experiments.

**Fig. 4.** Competitive inhibition of anti-DNase IgG binding to DNase or DNA by synthetic
peptides. Anti-DNase IgG (0.5 mg/ml) was pre-incubated with different amounts of DNase peptide (solid symbol) or control peptide (open symbol) for 1h at 37 ℃ and then transferred to DNase- (◆, □) or DNA- (●, ○) coated plates. Bound antibodies were detected as described in Material and Methods. Results are expressed as percent of inhibition.

**Fig. 5.** SDS-PAGE, Western blot and zymograph analysis of bovine DNase, human urine and streptococcal supernatants. (a) 12% SDS-PAGE, (b) Western blot, (c) zymograph. Lane 1: pre-stained molecular weight standard, lane 2: bovine DNase, lane 3: human urine, lanes 4 and 5: streptococcal supernatants from two different isolates.

**Fig. 6.** Inhibition of the enzyme activities of DNase from bovine, human and streptococcus by anti-DNase IgG and normal IgG. Different samples were pre-incubated with anti-DNase IgG or normal IgG at 37 ℃ for 1h, the remaining DNase activities were assayed as described in Materials and Methods. Data represent mean of duplicate.

**Fig. 7.** Anti-DNase and anti-DNA antibodies in NZB/NZW mice. NZB/NZW mice were immunized and boosted with DNase, as described in Materials and method. Sera were collected from both DNase immunized and control mice at different ages, as indicated.
Anti-DNase and anti-DNA antibodies in these sera were detected by DNase and DNA coated ELISA plates, as described in Materials and Methods. Data represent the mean ± SD of triplicate.

**Fig. 8.** Proteinuria in NZB/NZW mice. Urine from both DNase-immunized and control mice were collected at different ages as indicated. The amounts of protein in these urine were detected by BCA protein assay. Data represent mean ± SD of triplicate.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

(A) SDS-PAGE
Figure 5

(B) Western blot
Figure 5

(C) Zymograph
Figure 6

- Human urine (anti-DNase IgG)
- Human urine (normal IgG)
- Streptococal sup. (anti-DNase IgG)
- Streptococal sup. (normal IgG)
- Bovine DNase (anti-DNase IgG)
- Bovine DNase (normal IgG)

% of Inhibition
Figure 7
Figure 8