Induction of systemic lupus erythematosus-like syndrome in syngeneic mice by immunization with activated lymphocyte-derived DNA

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Objectives. Systemic lupus erythematosus (SLE) is the prototype of autoimmune disease and the mechanisms underlying the disease have not yet been elucidated. Thus, animal models of SLE would facilitate investigation of pathogenetic mechanisms involved in the development of the disease. This study characterizes a murine model of SLE-like syndrome induced by syngeneic activated lymphocyte-derived DNA (referred to as ALD DNA).

Methods. Normal BALB/c mice were immunized subcutaneously with highly purified ALD DNA. Anti-double-stranded DNA (anti-dsDNA) antibodies were determined by enzyme-linked immunosorbent assay. Other SLE-associated autoantibodies were examined by indirect immunofluorescence and anti-ENA (extractable nuclear antigen) profile assay. Pathological changes were analysed by light microscopy and electron microscopy. Kidney cryostat sections were viewed by immunofluorescence for the presence of glomerular IgG and C3 deposits. Proteinuria was measured by Coomassie brilliant blue assay.

Results. High levels of anti-dsDNA antibodies and other autoantibodies frequently appearing in SLE were detectable in the sera of ALD DNA-immunized mice. Glomerulonephritis and glomerular deposition of IgG plus C3 were observed in the kidney sections. Moreover, proteinuria was seen in the immunized mice.

Conclusions. SLE-like syndrome can be induced by ALD DNA in normal mice. This induced model may be useful for elucidating the mechanisms involved in autoimmunity to DNA and the development of SLE.

KEY WORDS: SLE, Induced model, Anti-dsDNA antibody, Syndrome.

Systemic lupus erythematosus (SLE) is a severe multisystem autoimmune disease serologically characterized by the production of a variety of autoantibodies [1]. Among these antibodies, antibodies to double-stranded DNA (dsDNA), especially those of the IgG isotype, are thought to be diagnostic markers in SLE and their presence in humans and mice often correlates with disease pathogenesis [2–5].

Although several factors of importance have been reported in the induction of autoantibodies and development of the disease [6], the pathogenesis of SLE is still unclear; hence the establishment of animal models could be of considerable value. Various spontaneous murine models that spontaneously develop a lupus-like syndrome, such as (NZB × NZW)F1, MRL/lpr and BXSB, have been described; however, each of them has a distinct, characteristic genetic makeup and immunological features [7]. Thus, induced models in healthy mice that are not genetically susceptible to autoimmunity would facilitate the investigation of mechanisms of antibody production and development of SLE. In the present study we used activated lymphocyte-derived DNA (ALD DNA) to trigger lupus-like syndrome in a murine strain which does not develop any spontaneous immune disorder. We found that, when immunized with ALD DNA, syngeneic BALB/c mice developed a SLE-like syndrome characterized by the major hallmarks of SLE. However, under the same conditions, unactivated lymphocyte-derived DNA (UnALD DNA) was inactive. This model induced by ALD DNA may be useful for elucidating the pathogenetic mechanisms contributing to disease development and for providing therapeutic strategies for SLE.

Materials and methods

Mice

Female BALB/c mice between 6 and 8 weeks old were purchased from the Center of Experimental Animals of Fudan University and housed in the pathogen-free mouse colony at our institution. All animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P.R. China, 1998) and the guidelines of the Shanghai Medical Laboratory Animal Care and Use Committee.

Preparation of spleen cells

Spleens of naive BALB/c mice were aseptically removed and teased on a nylon mesh immersed in chilled RPMI-1640 medium (Gibco) in a plastic dish. Cells that passed through a nylon mesh were washed twice with RPMI-1640. The erythrocytes were lysed with Tris-NH4Cl and the remaining cells were resuspended in RPMI-1640 with 10% fetal bovine serum (Gibco) containing 2 mM...
glutamine (Sigma), 100 U/ml penicillin G and 100 mg/ml streptomycin. Then, splenocytes were diluted to a final concentration of $2 \times 10^6$ cells/ml and cultured with or without concanavalin A (Con A; final concentration 5 $\mu$g/ml) for 48 h.

**DNA preparation**

Genomic DNAs from syngeneic Con A-activated or unactivated splenocytes were extracted and treated with S1 nuclease (TaKaRa, Japan) and proteinase K (Sigma) according to the manufacturer’s instructions, and then purified using the UltraPure™ genomic DNA purification kit, as recommended by the manufacturer (Shanghai SBS Genetech). DNA concentrations were determined by absorbance (A) measurement at 260 nm. The final $A_{260}/A_{280}$ for all the DNA preparations was >1.8. Less than 0.01 U/$\mu$g endotoxin was present in any of the DNA samples, based on a Limulus amoebocyte lysate assay (BioWhittaker, Walkersville, MD, USA).

**Animal immunization**

Syngeneic BALB/c mice were divided into several groups of 8–10 mice and actively immunized by subcutaneous injection on the back with 0.2 ml of an emulsion containing the indicated doses of ALD DNA or UnALD DNA (week 0) in phosphate-buffered saline (PBS) plus complete Freund’s adjuvant (CFA, Sigma). Eight to 10 controls received an equal volume of PBS or CFA plus PBS. Two booster immunizations at week 2 and week 4 consisted of DNA with incomplete Freund’s adjuvant. Mice were bled from retro-orbital sinus prior to immunization and at 2-week internals until 6 months after the final boost.

**ELISA**

Total and IgG isotype antibodies to ALD DNA were assessed using a previously described enzyme-linked immunosorbent assay (ELISA) with modification [8]. Briefly, 96-well proline-sulphate-pretreated polystyrene microtitre plates (Nunc, Denmark) were coated with DNA at 50 $\mu$g/ml and placed overnight at 4°C after incubation at 37°C for 2 h. The plates were then washed three times with PBS containing 0.05% Tween-20 (PBST). Mouse serum diluted 1:100 in PBST containing 10% calf serum and 5% goat serum was added to each well and incubated for 2 h at 37°C. The plates were again washed, followed by the addition of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (IgM, IgA or IgE) or IgG subclass (HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3) specific secondary antibodies (Southern Biotech, USA) diluted 1:4000 in PBST. The plates were then incubated for 20 min with a substrate solution of O-phenylenediamine (OPD) (Sigma) and 30% H$_2$O$_2$. The absorbances were measured at 490 nm on a microtitre plate reader (Southern Biotech, USA).

**Indirect immunofluorescence (IIF) assays**

For the antinuclear antibodies, sera from eight ALD DNA-immunized mice and eight control mice were assessed for immunofluorescence pattern. Indirect immunofluorescence assays were performed on HEp-2 cells (a human larynx carcinoma cell line) using an indirect immunofluorescence kit (Research Center for Rheumatic Disease, Shanghai Ren Ji Hospital, China) according to the manufacturer’s specification. Briefly, ethanol-fixed HEp-2 cells were incubated first with the sera at a dilution of 1:10 and then with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Slides were then incubated for 30 min at 37°C, washed and observed with a fluorescence microscope.

Analysis of results was performed according to the manufacturer’s specification.

**Determination of anti-ENA antibodies (western blotting)**

Sera from 10 ALD DNA-immunized mice and 10 control mice were assessed for the anti-ENA (extractable nuclear antigens) spectrum using an anti-ENA profile kit (Research Center for Rheumatic Disease, Shanghai Ren Ji Hospital, China) according to the manufacturer’s specification. This is virtually a western blotting technology for the differentiation of autoantibodies against cell nuclei. Analysis of results was performed according to the manufacturer’s specification.

**Pathological analysis**

Another group of 10 ALD DNA-immunized mice and 10 controls was set up to evaluate the development of glomerulonephritis and proteinuria. Tissues from mouse kidneys were prepared and stained with haematoxylin and eosin using standard procedures. Tissues were prepared for electron microscopy as previously described [9] and examined through a transmission electron microscope (Philips).

**Antibody elution procedures**

The glomerular isolation procedure was performed and the eluate of antibodies from five isolated glomeruli in each group was then treated with citrate buffer as described previously [10].

**Assays for proteinuria**

Proteinuria was measured with the Coomassie brilliant blue assay as described previously [11].

**Determination of IgG and C3 deposition in kidney**

Kidneys from 10 ALD DNA-immunized mice and 10 controls were frozen in dry ice, embedded with OTC medium and 5 $\mu$m sections were obtained. Sections were stained with FITC-conjugated goat anti-mouse IgG (Sigma) or FITC-conjugated rabbit anti-mouse C3 (Sigma). Immune complex deposits were observed by fluorescence microscopy.

**Detection of IgG in glomerular eluate**

Glomerular eluate from five ALD DNA-immunized or five control mice was examined for IgG deposition using an avidin-biotinylated horseradish peroxidase technique as described previously [12].

**Statistical analysis**

The statistical significance of the results was evaluated using the Mann–Whitney U-test. Student’s t-test was used when appropriate. All data are expressed as mean±S.D. and $P < 0.05$ was considered significantly different.

**Results**

**Induction of anti-dsDNA antibodies by immunization with ALD DNA**

To determine the ability of ALD DNA to induce the production of anti-dsDNA antibodies in vivo, groups of eight BALB/c mice were first administered 50 $\mu$g of ALD DNA or UnALD DNA in Freund’s adjuvant. In the control group, the mice were immunized with PBS or CFA plus PBS. As shown in Fig. 1A, there were
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immunized with ALD DNA (50 µg), UnALD DNA (50 µg), PBS or CFA. Sera obtained 4 weeks after the final immunization were examined by ELISA. (B) Mice were next immunized with various amounts of ALD DNA (0.6–20 µg) and UnALD DNA (20 µg). Sera collected at various times after the final immunization were tested by ELISA. All data shown in this figure are representative of at least six independent experiments. Data represent the mean OD 490 values and standard deviations for eight mice in each group. **P < 0.01.

significantly high levels of IgG anti-dsDNA antibodies in the sera from ALD DNA-immunized mice (8/8). In contrast, mice immunized with UnALD DNA (0/8) did not produce anti-dsDNA antibodies. Similarly, the mice immunized with PBS (0/8) or CFA plus PBS (0/8) failed to show induction of anti-dsDNA production.

Based on the results described above, we asked whether the anti-dsDNA responses would be dependent on the amount of ALD DNA. To address this issue, six groups of eight mice were treated with graded doses (0.6–20 µg) of ALD DNA. As the data in Fig. 1B indicate, 2 weeks after the final boost, all eight mice receiving 10 µg (8/8) or 20 µg (8/8) of ALD DNA developed high levels of anti-dsDNA antibodies. The anti-dsDNA responses elicited by these two doses of ALD DNA reached maximal levels comparable to those induced with 50 µg of ALD DNA (Fig. 1A) at week 4; they then declined slightly but remained stable for at least 20 weeks at higher levels after the final immunization (Fig. 1B and data not shown). Two out of eight mice (25%) receiving 5 µg of ALD DNA developed IgG anti-dsDNA antibody. However, none of the eight mice receiving less than 2.5 µg ALD DNA showed this response. Therefore, 10 µg of ALD DNA was the minimal dose sufficient for induction of anti-dsDNA antibodies. Control mice

and the mice receiving high mounts of UnALD DNA (20 µg), however, did not produce appreciable anti-dsDNA responses over the course of the experiments.

**Isotype analysis of anti-dsDNA antibody induced by ALD DNA**

To determine whether ALD DNA immunization could cause the production of other classes of Ig, sera from eight mice treated with ALD DNA or UnALD DNA were examined. The results in Fig. 2A show that ALD DNA primarily induced IgG anti-dsDNA antibodies but had no significant effect on IgM, IgA or IgE anti-dsDNA production in BALB/c mice. The IgG isotype in the sera of ALD DNA-immunized mice was next assessed. The predominant IgG isotype was IgG1, with lower amounts of IgG2a and IgG2b detected (Fig. 2B). The level of IgG3 anti-DNA was quite low. These data suggest that ALD DNA seems to preferentially induce an Th2-type response in vivo.

**Immunofluorescence pattern**

Sera from eight ALD DNA-immunized mice were studied for their immunofluorescence pattern, using IIF. As shown in Fig. 3b–f, sera from ALD DNA-immunized mice showed different immunofluorescence patterns. The patterns of immunofluorescence observed were homogeneous, cytoplasmic, nucleolar and peripheral, indicating generation of various antinuclear antibodies. The immunofluorescence pattern was predominantly homogeneous; this type of immunofluorescence pattern was observed...
in the sera of six of eight (75%) ALD DNA-immunized mice. The individual frequencies of other immunofluorescence patterns were as follows: cytoplasmic pattern, 37.5% (3/8); nucleolar pattern, 25% (2/8); peripheral pattern, 12.5% (1/8); and mixed (cytoplasmic and nucleolar) pattern, 25% (2/8). In contrast, sera from UnALD DNA-immunized mice and control mice showed negative immunofluorescence staining (Fig. 3a and data not shown).

**Induction of other autoantibodies induced by ALD DNA**

Besides DNA, other nuclear materials, such as Sm and ribonucleoprotein (RNP), consistently emerge as targets in SLE both in human patients and animal models [13]. To determine whether ALD DNA could also induce antibodies to other nuclear components, sera from another group of 10 ALD DNA-immunized mice and 10 UnALD DNA-immunized mice were tested for anti-ENA antibodies by western blotting. As shown in Fig. 4, individual ENA antibodies, such as Sm, SS-A, SS-B and rRNP, were detectable 6–8 weeks after the final immunization in the sera of ALD DNA-immunized mice. The individual frequencies were as follows: anti-SSA/SSB, 30%; anti-Sm, 20%; anti-rRNP, 30%. Nevertheless, all the sera from UnALD DNA-immunized mice and control mice were negative for anti-ENA antibodies. Taken together, these findings imply that immunization with ALD DNA promotes multiple autoantibody production.

**Pathological analysis of kidneys from ALD DNA immunized-mice**

Kidney tissues from another group of 10 ALD DNA-immunized mice were analysed by light microscopy for the presence of glomerulonephritis. Twenty weeks after the final boost, nine of 10 mice (90%) immunized with ALD DNA showed glomerular cell proliferation (Fig. 5Ab) and tubular dilation (Fig. 5Ad). By electron microscopy, fusion of foot processes in visceral epithelial cells (Fig. 5Ba) and mesangial cell proliferation (Fig. 5Bb) were seen in the kidneys of mice treated with ALD DNA. In contrast, no histological abnormalities were observed in UnALD DNA-immunized mice control mice (Fig. 5Aa and c, and data not shown).

**Detection of IgG and C3 deposition in kidneys of ALD DNA immunized-mice**

The pathogenesis of glomerulonephritis associated with SLE is commonly attributed to the deposition of immune complex consisting of DNA and anti-DNA in the glomerulus [5]. Therefore, kidney cryostat sections from 10 ALD DNA-immunized mice were viewed by immunofluorescence for IgG deposits. IgG deposits appeared in the kidneys of nine mice (90%) treated with ALD DNA (Fig. 6Ab–d). Only one mouse out of 10 that immunized with UnALD DNA was found to have weak IgG deposits. None of the control mice had glomerular deposits of IgG (Fig. 6Aa and data not shown). As shown in Fig. 6Ad, the pattern of immunofluorescence observed primarily was a lumpy accumulation within the glomerular mesangium. To further investigate whether IgG deposited in the kidney could bind to DNA, IgG was eluted from kidneys of ALD DNA-immunized mice. IgG eluates from kidney of ALD DNA-immunized-mice showed a high level of binding whereas eluates from UnALD DNA-immunized and control mice were negative for binding (Fig. 6B). This result confirmed that IgG deposits observed by immunofluorescence microscope were present in the ALD DNA-immunized kidneys. C3 deposition was also seen in the mesangium.
and glomerular capillary from mice treated with ALD DNA (Fig. 6Ca), but not in UnALD DNA-immunized and control mice (Fig. 6Cb and data not shown). These results indicate that immune complexes of DNA, anti-DNA and complement may mediate glomerulonephritis in ALD DNA-immunized mice.

**Development of proteinuria by ALD DNA immunization**

Nine out of 10 (90%) ALD DNA-immunized mice producing anti-dsDNA antibodies developed proteinuria (Fig. 7; 435 ± 28 μg/ml) by 8 months of age, whereas the mice immunized with UnALD DNA or CFA had low levels of protein in their urine (22 ± 7 and 23 ± 10 μg/ml, respectively). We considered that the development of proteinuria was probably due to glomerulonephritis.

**Discussion**

SLE may last for many years and lead to disability for work and limited life quality [14]. The diversity of clinical manifestations and disease phenotype, together with limited access to patient specimens, has made it difficult to study human lupus. However, the availability of experimental animal models may shed light on the pathogenesis of SLE.

Several murine models that spontaneously develop a syndrome resembling human SLE are available. However, in these models, genetic factors contribute to the development of the disease [7]. Previous studies from our laboratory have shown that Con A-activated lymphocytes can trigger anti-DNA production and immune complex deposits in the kidney of syngeneic mice [15]. To identify the exact elements from activated cells responsible for this response, in the present study we used DNA from Con A-activated syngeneic lymphocytes (ALD DNA) and tested its ability to trigger anti-dsDNA in vivo. We observed that the ALD DNA could induce an SLE-like syndrome in a mouse strain not susceptible to autoimmunity. When immunized with ALD DNA, syngeneic BALB/c mice produced lupus-like syndrome that included (i) high levels of anti-dsDNA antibodies and other autoantibodies associated with SLE, as demonstrated by analysis of the immunofluorescence pattern and an anti-ENA profile assay, and (ii) renal lesions, as evidenced by overt glomerulonephritis, renal IgG and C3 deposition and proteinuria. However, under the same conditions, UnALD DNA and CFA were inactive.
UnALD DNA amount of DNA used in immunization, because 10 ALD DNA on anti-dsDNA responses was dependent upon the autoimmune disease. In addition, we noticed that the effect of drive [23–26]. Our results suggest that ALD DNA may serve as antigen, targeted by antibodies that bear features of DNA antigen autoimmune disease SLE, DNA is considered a major auto-
origin [22].

DNA in SLE plasma is of endogenous rather than exogenous origin [22]. This is a new model of DNA-induced SLE-like syndrome, since ALD DNA is an autologous DNA. In the prototypical autoimmune disease SLE, DNA is considered a major auto-
targeted by antibodies that bear features of DNA antigen drive [23–26]. Our results suggest that ALD DNA may serve as a driving antigen for autoantibodies and the development of autoimmune disease. In addition, we noticed that the effect of ALD DNA on anti-dsDNA responses was dependent upon the amount of DNA used in immunization, because 10 µg or a higher dose of ALD DNA could elicit anti-dsDNA antibodies. It seems likely that an abundant source of ALD DNA leads to a breakdown of self-tolerance of autoimmunity to DNA.

Our study also showed that normal BALB/c mice could produce autoantibodies under the appropriate conditions. Some studies have shown that DNA complexed with a protein carrier or a peptide in an adjuvant can induce anti-DNA antibodies and/or renal pathological changes in normal BALB/c mice [27–30]. Compared with their studies, our ALD DNA-induced SLE-like model had unique features: (i) the DNA used in our study is an autologous DNA rather than exogenous DNA; and (ii) ALD DNA without a DNA binding protein is sufficient to induce a lupus-like syndrome.

Kuroda et al. [31] have recently reported that a single intraperitoneal injection of the adjuvant oil pristane, incomplete Freund’s adjuvant or squalene induces lupus-associated autoantibodies in non-autoimmune BALB/c mice, suggesting an important role of adjuvant-induced autoimmunity. Therefore, CFA-immunized mice were used as one of the controls in our experiment. Anti-
dsDNA (Fig. 1B) and anti-ENA antibodies (Fig. 4) were not detectable in the CFA-immunized mice. However, we could not completely rule out the role of adjuvant in the induction of autoantibodies in this model because ALD DNA alone, i.e. without CFA, did not elicit anti-dsDNA antibodies (data not shown).

It should be noted that ALD DNA-immunized mice showed an early onset of high levels of IgG anti-dsDNA antibodies at 14–16 weeks of age, while NZB/NZW mice have been reported spontaneously to produce anti-dsDNA antibodies at 26–30 weeks of age [32]. Serum IgG isotype analysis of ALD DNA-immunized mice revealed that IgG1 was the predominant anti-
body, indicating a Th2-type response. It is possible that Th2-type cytokines resulted in a markedly increased frequency of B cells secreting pathogenic autoantibodies, leading to accelerated disease in non-autoimmune mice. Further experiments will be necessary to address this issue.

Besides anti-dsDNA, antibodies to widely expressed intracellular components such as ENA were also detectable in ALD DNA-immunized mice. We observed that development of autoantibodies to ENA followed the initiation of an immune response to DNA. Anti-ENA antibodies emerged 6–8 weeks after the final immunization, while anti-dsDNA antibodies appeared as early as 2 weeks after the final immunization. Epitope spreading is likely to account for the appearance of a variety of antinuclear antibodies in sera from ALD DNA-immunized mice.

In conclusion, syngeneic BALB/c mice immunized with ALD DNA responded by developing high levels of anti-dsDNA antibodies. The immunized mice producing anti-dsDNA antibo-
odies also developed proteinuria, which could result from induction of glomerulonephritis. This view is supported by the IgG deposits and eluates from the kidney of ALD DNA-
immunized mice. This new model of SLE-like syndrome would not only be helpful for understanding the trigger for autoimmunity to DNA and pathogenetic mechanisms of SLE, but also for developing effective therapeutic strategies for the disease.

![Fig. 7](https://academic.oup.com/rheumatology/article-abstract/44/9/1108/1784352)

**Fig. 7.** Urine protein induced in ALD DNA-immunized mice. Urine specimens were collected from mice of each group and urine protein levels were measured. The figure shows the mean values for 10 mice in each group. **P < 0.01.

One of the most likely explanations for these findings is that ALD DNA can be recognized by the murine immune system due to its unique conformation or certain modified bases rarely present on UnALD DNA. ALD DNA and UnALD DNA might differ in the frequency and methylation of the Cpg motif since Cpg dinucleotides are under-represented (Cpg suppression) and selectively methylated at the 5-position of the cytosine in vertebrate DNA [16]. Interestingly, we have found that the methylation extent of 5-cytosine in DNA from Con A-activated splenocytes is lower than that of DNA from normal cells [17]. It has been observed that hypomethylation of DNA regulatory sequences is involved in activation of B and T lymphocytes [18, 19]. Therefore, we hypothesized that hypomethylated cytosines may contribute to the immunogenicity of ALD DNA. In fact, there is a situation in which hypomethylated DNAs have emerged in patients with SLE [20, 21]. This interpretation is consistent with evidence that DNA in SLE plasma is of endogenous rather than exogenous origin [22].

Our study also showed that normal BALB/c mice could produce autoantibodies under the appropriate conditions. Some studies have shown that DNA complexed with a protein carrier or a peptide in an adjuvant can induce anti-DNA antibodies and/or renal pathological changes in normal BALB/c mice [27–30]. Compared with their studies, our ALD DNA-induced SLE-like model had unique features: (i) the DNA used in our study is an autologous DNA rather than exogenous DNA; and (ii) ALD DNA without a DNA binding protein is sufficient to induce a lupus-like syndrome.

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