Induction of systemic lupus erythematosus syndrome in BALB/c mice by immunization with active chromatin[1]

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ABSTRACT

AIM: To establish an animal model for systemic lupus erythematosus (SLE)-like syndrome in mice. **METHODS**: BALB/c mice were immunized with active chromatin isolated from ConA-actived syngeneic spleno-lymphocytes. Plasma samples of mice were tested by enzyme-linked immunosorbent assays (ELISA) for the presence of IgG anti-dsDNA, -ssDNA, and anti-histone antibodies. Tumor necrosis factor-α (TNF-α) in serum was measured by ELISA. Spleno-lymphocyte proliferation assays and the levels of interferon-γ (IFN-γ) in supernatants were tested respectively. Proteinuria was measured. Kidneys were examined by direct immunohistochemical method and light microscopy. **RESULTS:** Anti-ds DNA, ssDNA, and histone antibodies were induced in active chromatin-immunized mice, the proliferation response of splenocytes to ConA and LPS were reduced, levels of interferon-γ in supernatants and TNF-α in serum were lowered. Lupus nephritis was assessed by the presence of Ig deposits, glomerular pathology and proteinuria. **CONCLUSION:** The active chromatin-induced SLE-like mouse model was similar to idiopathic SLE in human.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease which is characterized by the production of autoantibodies to nuclear proteins and nucleic acids, accompanied with clinical manifestation (such as leukopenia and thrombocytopenia) and kidney damage. The pathogenesis of SLE is thought to be primarily under genetic control, with environmental factors playing a secondary role. Human SLE is influenced strongly by major histocompatibility complex-linked and nonlinked genes. SLE occurs spontaneouly in certain strains of mice, such as NZB/W(F1) and MRL/pr with multiple genetic loci that accelerate the onset of the disease[1,2]. The importance of environmental factors in the pathogenesis of SLE is less clear. It remains uncertain what antigen triggers the production of autoantibodies as mammalian double strand DNA (dsDNA) is poorly immunogenic. SLE like syndrome could also be induced in BALB/c by intraperitoneal injection of pristane, a chemical component. As BALB/c mice is a strain not usually considered to be predisposed autoimmunity, SLE may be produced by an interplay of genetic and environmental factors[2,3]. We have found that active DNA could induce anti-nuclear antibodies and renal damage in BALB/c mice[4]. In this study, we try to understand the basis for the loss of self-tolerance in BALB/c mice following immunization with active chromatin and to

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determine the validity of this system as a useful model for SLE.

MATERIALS AND METHODS

Mice Female BALB/c mice 6-wk-old were purchased from Sino-British Sippr/BK Lab Animal Ltd (Grade SPF II Certificate No 153). Mice were housed in the specific pathogen free condition.

Antigen BALB/c mice spleen cells were cultured with RPMI-1640 medium containing 10 % fetal calf serum. Activated spleen cells were stimulated by ConA (5.0 mg/L), while non-activated spleen cells were kept in a mitogen-free medium. After 48 h, the cells were harvested and treated as Zhong *et al*[5] to get active and resting chromatin.

Immunization Mice were immunized with 100 μg active or resting chromatin in 200 μL Freund's complete adjuvant (containing BCG 10 g/L) on d 0 and then boosted in Freund's incomplete adjuvant on d 14 and chromatin suspension on d 28. Each mouse received 200 μL of the cold emulsion by intradermal (id) injection on back, 1 or 2 injections into the base of the tail. Mice were sacrificed on d 45 or d 60.

Assays for serum antibody For the detection of specific antibodies in sera of immunized mice, ELISA was carried out as described previously[6]. Murine serum was diluted at 1:200 and peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies was diluted at 1:2000 (Huamei Company). For the detection of anti-dsDNA antibodies and anti-ssDNA antibodies, 96-well plates (Costar, Corning, NY) were coated with calf thymus DNA (Sigma) or denatured calf thymus at 50 g/L. The histone from calf thymus (Sigma) was used at 10 mg/L for detection of anti-histone antibodies. Optical density was monitored at 492 nm using a well scan ELISA reader (Labsystems Dragon). Results were indicated in Enzyme Index (EI). EI $=100\times OD_{\text{Tested group}}/(OD_{\text{nil group}}+3\text{SD})$.

Spleno-lymphocyte proliferation assays[7,8] On d 60 spleen cells of immunized mice were harvested and plated at 1×10^6–2×10^6 cell/well in 96-well U-bottom plates in RPMI-1640 medium supplemented with 10 % fetal calf serum. Some cells were pulsed with ConA at 6 mg/L or LPS at 10 mg/L in duplicate for 48 h. A stock solution of MTT (Sigma) 5 g/L was added as 20 μL/well, and the microplates were incubated at 37 °C for 4 h. The plates were centrifuged at $800\times g$ for 20 min) and the supernatants were carefully discarded. Me_2SO 150 μL was added to each well, then the absorbance were read on well scan (Labsystems Dragon) at 492 nm. Results were indicated in stimulation index (SI). $SI=OD_{\text{without mitogen}}/OD_{\text{with mitogen}}$.

Measurement of TNF-α and IFN-γ On d 60 murine serum was isolated. TNF-α in serum and IFN-γ in spleno-lymphocyte supernatant was determined with ELISA kits (Diaclone) based on standard curves.

Proteinuria assays[4] Proteinuria was measured by Coomassie brilliant blue assay. Albumin (bovine serum) was used as standard curves. Murine urine were centrifuged (20 min, 4500 rpm), and supernatants were taken and diluted at 1:10 with normal saline (NS). After Coomassie brilliant blue solution were added, the sample were tested at 540 nm and the concentration of proteinuria could be calculated by standard curves.

Renal pathology and deposition of Ig One kidney from each animal was fixed in 10 % formaldehyde and embedded in paraffin. Sections of 5-μm-thick were cut and stained with hematoxylin and eosin (H&E) for light microscopy.

The 5-μm section were deparaffinized, rehydrated, and stained for 10 min with peroxidase-conjugated goat anti-mouse Ig (Two-step anti-mouse detection reagent (HRP) kit, Antibody Diagnostic Inc) for the assessment of kidney Ig deposition.

Statistical analysis Data were expressed as mean±SD and compared by the ANOVA and Fisher's PLSD test.

RESULTS

IgG anti-ds-DNA, anti-ss-DNA, and anti-histone autoantibodies Anti-nuclear antibodies are associated with lupus nephritis in humans. Active chromatin-treated mice had markedly elevated levels of IgG anti-ds-DNA, anti-ss-DNA, and anti-histone when tested at d 44 and d 60. In nil and resting chromatin groups no significant anti-nuclear antibody response was found in BALB/c mice, a "non-autoimmune" strain (Tab 1).

Spleno-lymphocyte proliferation In the absence of mitogen, the spleno-lymphocyte proliferation was stronger in the active chromatin-immunized group than those in nil and resting chromatin-immunized groups on d 45 and d 60 ($P<0.01$). But after LPS or Con A stimulation, the spleno-lymphocyte proliferation was decreased in the active chromatin-immunized group compared with nil and resting chromatin-immunized

Tab 1. Induction of IgG anti-dsDNA, ss-DNA, histone antibodies. *n*=6. Mean±SD. [a]*P* >0.05, [b]*P*<0.05,[c]*P*<0.01 *vs* resting chromatin. [d]*P* >0.05, [e]*P*<0.05, [f]*P*<0.01 *vs* nil.

	Nil	Resting chromatin	Active chromatin
Anti-dsDNA			
d 0 (*n*=12)	56±14	48±14[d]	49±13[ad]
d 45	59±14	59±17[d]	80±10[be]
d 60	69±10	73±6[d]	94±6[cf]
Anti-ssDNA			
d 45	66±11	72±12[d]	87±8[bf]
d 60	72±9	78±10[d]	108±9[cf]
Anti-histone			
d 45	77±8	85±12[d]	118±22[cf]
d 60	74±9	77±14[d]	99±15[bf]

Tab 2. Comparison of mitogen induced spleno-lymphocyte proliferation. *n*=6. Mean±SD. [a]*P* >0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* resting chromatin; [d]*P* >0.05, [e]*P*<0.05, [f]*P*<0.01 *vs* nil.

	Nil	Resting chromatin	Active chromatin
d 45 (2×10^6cells/well)			
Absence of mitogen ($OD_{492\ nm}$)	0.90±0.05	0.88±0.04[d]	1.01±0.05[cf]
SI (ConA)	2.08±0.15	2.09±0.15[d]	1.86±0.14[be]
SI (LPS)	1.48±0.16	1.45±0.11[d]	1.1±0.8[cf]
d 60 (1×10^6cell cells/well)			
Absence of mitogen ($OD_{492\ nm}$)	0.23±0.02	0.22±0.02[d]	0.27±0.02[cf]
SI (ConA)	4.3±0.6	4.3±0.57[d]	3.4±0.22[cf]
SI (LPS)	1.33±0.17	1.43±0.14[d]	1.2±0.12[c]

group (P<0.05, P<0.01, Tab 2).

Concentration of TNF-α, interferon-γ, and proteinuria Levels of TNF-α in serum and interferon-γ in supernatant was decreased in resting and active chromatin-immunized group, especially in the latter. The active chromatin inoculated mice had elevated levels of protein in their urine compared with other two groups.

Pathogenicity Active chromatin immunized mice all had moderate to severe renal Ig deposition, mostly localized to glomeruli, but no glomerular Ig deposition was present in mice in control group (Fig 1). Under light microscopy kidney sections from active chromatin immunized mice showed segmental or diffuse proliferative glomerular lesion. Proliferation was mainly related to increase in mesangial cells. In addition, mild influx of monocytes was seen in the glomerular capillaries. These changes were not apparent in the glomeruli of control mice (Fig 2).

Tab 3. Concentration of TNF-α in serum, IFN-γ in supernatants and proteinuria in urine at d 60. *n*=6. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* resting chromatin; [d]*P*>0.05, [e]*P* <0.05, [f]*P*<0.01 *vs* nil.

	Nil	Resting chromatin	Active chromatin
IFN-γ/ng·L^{-1}	47±8	30±5[f]	17±12[ef]
TNF-α/ng·L^{-1}	116±20	84±26[e]	17±6[cf]
Proteinuria/g·L^{-1}	0.8±0.2	0.7±0.2[d]	1.0±0.1[ce]

DISCUSSION

We report here the establishment and characterization of active chromatin-induced SLE-like syndrome in BALB/c mice, a strain not usually considered to be genetically susceptible to induce lupus. The syndrome induced by active chromatin shares many of the characteristics of human lupus including both clinical features (glomerular hypercellularity, glomerular Ig deposition, proteinuria) and the autoantibody profile (anti-ds-DNA, -ssDNA, histone), with complex immune abnormality[1-3].

After four batches of mice were immunized with active chromatin, autoantibodies and glomerular lesion could be found in all these mice (30/30). During the disease forming process, the anti-ds DNA antibodies could be detected early at d 18, anti-ss DNA at d 45, and anti-histone at d 32. Here we only show the results from one of these batches at d 45 and d 60. Anti-DNA antibodies have been shown to cause lupus-like nephritis in mice[1-3]. Ig deposition, renal proteinuria, and histological changes in active chromatin-immunized mice indicated that the high levels of auto-antibodies were pathogenic.

In untreated SLE patients, T, B lymphocytes as well as monocytes has immunoregulatory disturbance[9]. Impaired responses to mitogen were also observed in MRL/lpr mice, a genetically determined mice model for SLE. These mice lack an important cell death receptor

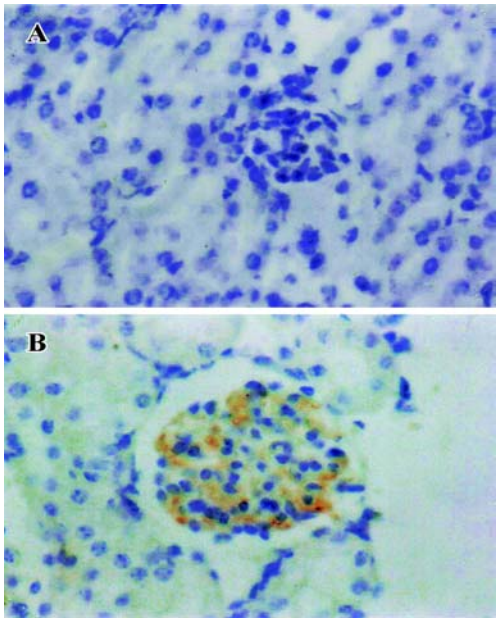

Fig 1. Immunohistology of kidney section from immunized mice on d 60. Glomerular Ig deposition was present in active chromatin immunized BALB/c mice. Kidney sections were stained with peroxidase-conjugated goat anti-mouse Ig followed by substrate. A: resting chromatin-immunized mouse. B: active chromatin-immunized mouse with marked capillary and mesangial Ig deposits (yellow). ×200.

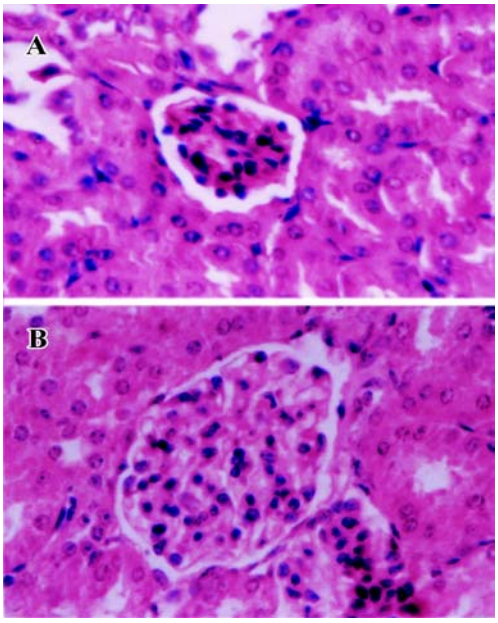

Fig 2. Renal morphology under light microscopy on d 60. H&E stain. A: resting chromatin-immunized mice with normal glomeruli. B: active chromatin-immunized mice with extensive endocapillary hypercellularity, thickening of the capillary walls, obliteration of capillary lumens and segmental sclerosis (note glomerular enlargement). ×200.

Fas, and accumulate large amount of dysfunctional lymphocytes, most of them are $CD4^-CD8^-$ T lymphocytes[7]. The higher spleno-lymphocyte proliferation in the absence of mitogen but lower response after Con A or LPS stimulation in active chromatin-induced mice may be a result of a large number of non-functional lymphocytes production. But this still needs to be proofed in further research.

The importance of T_{H1} and T_{H2} cytokines in lupus is controversial, perhaps reflecting heterogeneity of the disease. Altered cytokine homeostasis is a feature common to human and experimental lupus. It has been proposed that SLE is mediated by T_{H2} cytokines such as IL-10 and IL-4, but the situation is less clear in murine lupus[3]. It appears that both T_{H1} and T_{H2} cytokines can play an important role in disease pathogenesis. It is believed that TNF-α suppresses humoral autoimmunity, and induce IFN-γ production. IFN-γ enhance cytotoxic T lymphocyte (CTL) function by up-regulating Fas and FasL expression that would suppress humoral autoimmunity by killing autoreactive B cells. Recently, agents that neutralize TNF-α in some patients can cause surprising side effect including development of humoral autoimmunity and even clinical features of SLE, therapeutic TNF-α blockade may promote humoral autoimmunity by selectively inhibiting the induction of CTL response that would normally suppress autoreactive B cells[10,11]. In our experiment, TNF-α concentration in serum and IFN-γ production in splenocyte were mildly reduced in resting chromatin immunized group, but markedly reduced in active chromatin treated mice. The lowered production of IFN-γ may relate with the large numbers of non-functional lymphocytes in spleen. The changes in cytokine levels may induce or exacerbate disorders of humoral autoimmunity in our animal model. More experiments need to be done to find out that T_{H1}-T_{H2} balance is influenced and the balance could be an important determinant of renal disease following active chromatin exposure.

Environmental factors and genetic background play important roles in SLE induction[3]. There are mainly two kinds of animal models for SLE study, one occurs spontaneously in certain strains of mice such as NZB/(F1) and MRL/lpr with special genetic background[12], another can be induced in strains that are not usually considered to be predisposed autoimmunity. The spontaneous SLE model has its deficits: long latent time (over 6 months), the disease onset and the rate are not identical, and the genetic deficits do not fit for most

patients. The induced SLE-like syndrome in non-autoimmune mice strains is often used to investigate the causes for the broken of immune tolerance[1,2,11] since these various experimental SLE animal models represent various aspect of SLE. As the concerning factors inducing SLE could directly or indirectly activate lymphocytes, it is possible that the change of the antigenicity of chromatin is the common pathway to SLE pathogenesis, as we observed in our animal model. Compared with other SLE animal models, the syndrome appeared more fast (<60 d) and identical and mimic SLE in many aspects, so this model can serve as an useful tool for the investigations of mechanism and experimental therapies.

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