BIOLOGICAL ACTIVITIES OF DEOXYSPERGUALIN IN AUTOIMMUNE DISEASE MICE

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An assessment of the prophylactic and ameliorative effects of deoxyspergualin (NKT-01), an immunosuppressive agent, was carried out in male MRL/MpJ-lpr/lpr (MRL/1) mice which spontaneously develop lupus-like lesions. When NKT-01 was administered ip daily from the age of either 8 or 19 weeks, diseases such as massive lymphadenopathy, circulating anti-DNA antibody and lupus nephritis were markedly suppressed. The primary response to lipopolysaccharide was significantly reduced in MRL/1 mice administered NKT-01 but the response to sheep red blood cells was not affected. The ability of spleen cells to release interleukins 2 and 3 with or without mitogen was significantly enhanced in mice receiving NKT-01. These findings demonstrate that NKT-01 has therapeutic activity against the development of spontaneous disease in MRL/1 mice.

Deoxyspergualin (NKT-01) is a derivative of spergualin, a metabolite of *Bacillus laterosporus*[1] shown to have immunosuppressive activities in rodents[2]. This derivative, with a molecular weight of 496.9 and a moiety bearing a spermidine and a guanidinic group, has been found to suppress antibody production and delayed-type hypersensitivity to sheep red blood cells (SRBC) and to have a stronger activity than spergualin in inhibiting the rejection of skin allografts in rats[3]. In mice, NKT-01 inhibits the development of graft-versus-host disease[4]. In rats, NKT-01 inhibits the rejection of heart[5], liver[6], kidney[7] and pancreas[8] allografts. The purpose of the present study is to examine whether NKT-01 can prevent the spontaneous development of autoimmune disease and affect the immune response, when administered to male MRL/MpJ-lpr/lpr (MRL/1) mice.

Materials and Methods

Animals

Male MRL/1 and MRL/MpJ-+/+ (MRL/+) mice were purchased from Jackson Laboratories, Bar Harbor, U.S.A.

Treatment

NKT-01 was supplied by Takara Shuzo Co., Ltd., dissolved in saline and sterilized by passing through a 0.22-μm filter. In a previous experiment, when NKT-01 was administered ip daily at an immunosuppressive dose of 3 mg/kg from week 12, weight loss was observed about 6 weeks after the initial administration of NKT-01. Therefore, the dose was lowered to 1.5 mg/kg in all experiments.

Measurement of Anti-DNA Antibody by Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was carried out using modified methods of EATON *et al.*[9] and GODFREY *et al.*[10]. An aliquot (0.3 ml) of 0.05% aqueous solution of poly-L-lysine was incubated in a Nunc ELISA plate for 1.5 hours at room temp. The plates were washed using phosphate buffered saline with 0.1% gelatin (PBS-G) as the washing and diluting buffer, and 0.2 ml of calf thymus DNA (5 μg/ml PBS-G; Sigma, St. Louis, U.S.A.) was added. After incubating overnight at 37°C, any free sites were blocked by incubating with 0.2% bovine serum albumin for 1 hour at 37°C. A 0.2-ml aliquot of an 1/200-dilution of each mouse serum was then added and the plates were incubated for 1.5 hours at 37°C. The plates were then washed, and 0.2 ml of an 1/1,000-dilution of goat anti-mouse IgG serum (Cappel, Cochranville, U.S.A.) was added and incubated for 1.5 hours at 37°C. After washing, an 1/1,000-dilution (0.2 ml) of alkaline phosphatase-conjugated rabbit anti-goat IgG serum (Cappel) was added and incubated 1.5 hours at 37°C. Finally, 0.2 ml of *p*-nitrophenyl phosphate (Sigma), 2 mg/ml in 10% diethanolamine buffer, pH 9.8, was added and after 1 hour incubation at room temp, the absorbance of *p*-nitrophenyl was measured at 410 nm.

Blood Urea Nitrogen (BUN)

BUN was estimated by the urease-indophenol method using a Rapid Blood Analyzer Super (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

Histology

Kidneys were fixed in 10% buffered formalin and sectioned. The sections were stained with hematoxylin-eosin, periodic acid shiff, Masson and fibrin-staining methods for histopathological examinations. Twenty-five glomeruli were observed per section and the degree of glomerular damage was indicated by a pathological score based on the severity of change where 0=no, 1=minimal, 2=slight, 3=moderate and 4=severe.

Plaque-forming Cells (PFC) Assay

PFC producing antibody to SRBC (Nippon Biosupp Center, Tokyo, Japan) and *Escherichia coli* 055 : B5 lipopolysaccharide (LPS; Difco, Detroit, U.S.A.) were enumerated according to CUNNINGHAM and SZENBERG[11]. PFC producing anti-LPS were measured using LPS-coated SRBC as indicator cells.

Production of Interleukins 2 (IL 2) and 3 (IL 3)

RPMI 1640 medium supplemented with 10% fetal calf serum, streptomycin (100 μg/ml) and benzylpenicillin (100 U/ml) was used to incubate spleen cells from each MRL/1 mouse at 37°C in 5% CO_2 in air. To prepare culture supernatants containing IL 2 and IL 3, the spleen cells (5×10^6 cells/ml/well) were incubated with or without 2.5 μg/ml of concanavalin A (Con A; Sigma) in 24-well Costar culture plates. After 48 hours incubation the supernatants were separated. The presence of IL 2 and IL 3 was assayed by the ability of the supernatants to support proliferation of IL 2-dependent cell line CTLL-2[12] and IL 3-dependent cell line FDC-P2[13] that was provided by Dr. K. KUMAGAI, Tohoku University, Japan, respectively.

Statistical Analysis

All data were analyzed by Student's t-test.

Results

MRL/1 mice spontaneously develop massive lymphadenopathy, circulating antinuclear antibody, increased BUN and glomerulonephritis. Results obtained in mice that were 8 weeks old at the beginning of the prophylactic study of NKT-01 are shown in Table 1. At the age of 22 weeks and completion of the NKT-01 administration, all parameters were significantly suppressed in mice receiving NKT-01. In contrast, there was an insignificant decrease in body weight between control mice (mean=49.1 g) and NKT-01 treated mice (mean=48.5 g).

In a second series of experiment, we examined the curative use of NKT-01. NKT-01 was ad-

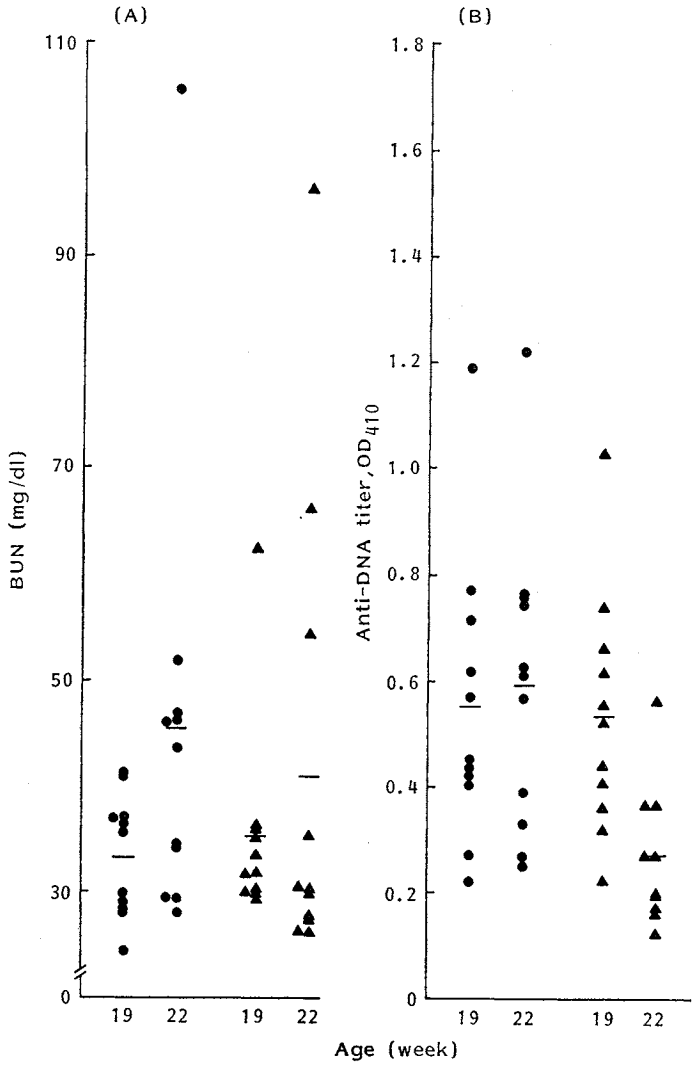
Table 1. Prophylactic activity of NKT-01 on the development of lupus-like lesions in MRL/1 mice.

Group	*n*	Lymph node (mg)	Spleen (mg)	Anti-DNA titer (A_{410} nm)	BUN (mg/dl)	Degree of glomerular damage (score)
Control	8	3,654±1,418	528±71	0.80±0.30	33±7	1.5±0.5
NKT-01	9	483±68*	166±42*	0.35±0.15*	19±2*	0.3±0.4*

NKT-01 was administered ip daily for 4 weeks followed by a 1-week interval and this was repeated 3 times from week 8 through 22. During the 2 intervening weeks no NKT-01 was given. A set of control MRL/1 mice receiving saline was included. The mice were sacrificed on the day after completion of the NKT-01 administration and lymph nodes, spleens, blood and kidneys were removed. The mesenteric, axillary, elbow, inguinal, submaxillary lymph nodes were pooled individually. Data are shown as mean± SD.

* $P<0.01$.

Fig. 1. Changes of BUN (A) and circulating anti-DNA levels (B) in control (●) and NKT-01 administered MRL/1 mice (▲).



The testing conditions were the same as those described in Table 2.
Each bar shown is a mean value.

Table 2. Suppression of advanced, spontaneous lymphadenopathy and lupus nephritis in MRL/1 mice by NKT-01.

Agent	*n*	Lymph node (mg)	Spleen (mg)	Degree of glomerular damage (score)
Saline	11	3,583±1,512	601±287	3.0±0.6
NKT-01	11	1,814±634**	358±158*	2.2±0.8*

NKT-01 was administered ip daily for 24 days from week 19 through 22. The other testing conditions are the same as those described in the legend of Table 1. Data shown are means with SD.
* $P<0.05$, ** $P<0.01$.

ministered daily for 24 days from week 19 through 22. Control mice had a mean BUN level of 33.4 mg/dl at week 19 and their levels rose significantly ($P<0.05$) to a mean of 45.5 mg/dl at week 22 (Fig. 1A). In contrast, an nonsignificant increase in the BUN level was observed in NKT-01 treated mice (an initial mean of 35.2 mg/dl; a final mean of 41.0 mg/dl). However, after this 24-day regimen, an abnormal BUN was seen only in a few control mice and the number of mice with an abnormal BUN score was similar in NKT-01 treated mice. The NKT-01 treated mice had a mean anti-DNA titer of 0.53 at week 19 which significantly ($P<0.005$) decreased to a mean of 0.27 by week 22 (Fig. 1B). This reduced level of anti-DNA titer at 22 weeks in treated mice was also significantly ($P<0.005$) less than that of control (mean=0.59) (Fig. 1B).

Table 3. Effect of NKT-01 on antibody production to SRBC and LPS in MRL/1 mice.

Antigen	Agent	PFC number/ 10^6 spleen cells	
		MRL/1	MRL/+
SRBC	Saline	137±140	1,383±383
	NKT-01	125± 51	1,125±376
LPS	Saline	101± 14	109± 19
	NKT-01	2± 4*	4± 3*

MRL/1 and MRL/+ mice of 10-week old were used. Each group consisted of 5 mice. NKT-01 or saline was administered ip daily until the day before the PFC assay. SRBC (10^8) were injected iv into mice 32 days after the initial administration of saline or NKT-01. Four days later spleen cells were removed and assayed for PFC producing anti-SRBC. LPS (30 mg) was injected iv into mice 33 days after the initial administration of saline or NKT-01. Four days later spleen cells were assayed for anti-LPS PFC. Data are shown as mean±SD.
* $P<0.01$.

Table 4. Augmented ability of spleen cells from NKT-01 treated MRL/1 mice to release IL 2 and IL 3.

Agent	*n*	IL 2 activity		IL 3 activity	
		Without Con A	With Con A	Without Con A	With Con A
Saline	5	2,883± 320	3,658±1,207	5,532±1,211	49,536±4,152
NKT-01	3	11,493±1,841*	82,146±3,096*	25,325±1,397*	74,207±7,243*

Ten-week old MRL/1 mice were used. NKT-01 was administered ip daily for 42 days. The day after completing NKT-01 administration spleens were removed. Both IL 2 and IL 3 activities in supernatants from the spleen cells with or without Con A stimulation were quantitated in the presence of 1×10^4 CTLL-2 or FDC-P2 cells in a final volume of 0.2 ml. These cells were incubated for 24 hours, pulsed with 1 μCi of [6-^{3}H]thymidine ([^{3}H]TdR, New England Nuclear, Boston, U.S.A.), and further incubated for 6 hours. After the cells were harvested, measurement of [^{3}H]TdR incorporation was carried out in liquid scintillation counters. Data corresponding to 1/4 dilution of the supernatants are shown as mean cpm with SE. The background levels of [^{3}H]TdR incorporation into CTLL-2 or FDC-P2 cells were a mean cpm of 535 and 506, respectively.
* $P<0.01$.

Similarly, total lymph node and spleen weights, and the degree of glomerular damage were significantly reduced by the administration of NKT-01 to these mice (Table 2).

As shown in Table 3, control MRL/1 mice showed poor primary response to SRBC and normal response to LPS compared with nonautoimmune MRL/+ mice. We examined whether NKT-01 could affect these responses in MRL/1 mice. NKT-01 did not affect the primary response to SRBC but significantly inhibited antibody production against LPS.

The spleen cells from control MRL/1 mice released little IL 2 and abnormal amounts of IL 3 in the presence or absence of Con A (Table 4). The ability to release IL 2 and IL 3 of spleen cells with or without Con A was significantly enhanced by the NKT-01 administration.

Discussion

The prophylactic administration of NKT-01 with ip doses of 1 mg/kg has been shown to prevent organ rejection in various allo-transplantation models and experimental allergic encephalomyelitis in guinea pigs[14]. Therefore, we predicted that the treatment of 8 week old MRL/1 mice with this drug might prevent the appearance of their autoimmune diseases. Although the prophylactic administration of NKT-01 at an immunosuppressive dose of 3 mg/kg induced severe body weight loss, NKT-01 at a lower dose of 1.5 mg/kg used here markedly inhibited the development of their autoimmune diseases without toxicity. Of particular interest was whether this immunosuppressive agent could stop or ameliorate autoimmune disease in MRL/1 mice suffering advanced spontaneous diseases. Those animals which initially had high anti-DNA titer showed significantly decreased levels, when given NKT-01 for only 24 days from the age of 19 weeks. The NKT-01 therapy also resulted in decreased glomerular damage. The effectiveness of NKT-01 in decreasing the anti-DNA titer was further substantiated by a suppression of antibody production to the T-independent antigen LPS. Since the administration period of NKT-01 was only 24 days in the present curative study, its long-term administration needs to be examined to determine the prolonged amelioration of autoimmune diseases and an extension of life span.

Control MRL/1 mice have also been shown to manifest not only a poor primary response to SRBC[15] but also a defective ability to release IL 2 after Con A stimulation[16,17]. Although NKT-01 did not affect antibody production to SRBC, both spontaneous and Con A-stimulated IL 2 release were enhanced in MRL/1 mice receiving NKT-01. Significantly, the defective ability of IL 2 release following Con A stimulation was effectively restored by NKT-01 administration. PALACIOS proposed that an abnormal production of IL 3 accounted for the outgrowth of nonmalignant T cells responsible for lymphadenopathy in MRL/1 mice[18]. Since, in the present studies, NKT-01 led to a significant reduction in lymphadenopathy in MRL/1 mice, we naturally expected that this agent would suppress IL 3 release. Contrary to our expectation, a significant enhancement of IL 3 release with or without Con A was observed. Therefore, NKT-01 is suggested to suppress lymphadenopathy independently of IL 3 production.

As NKT-01 very rapidly hydrolyzes under usual culture conditions, it is difficult to examine the mode of NKT-01 action *in vitro*. At present, there is no evidence showing an *in vitro* effect of NKT-01 at physiological concentrations (<1 μg/ml). Recently, DICKNEITE *et al.* demonstrated that NKT-01 administration suppresses the chemiluminescence reaction of blood monocytes and not the blastogenic response of blood lymphocytes in rats[19]. We found that NKT-01 enhances the function of spleen cells in the blastogenic response, or in the release of IL 1 and IL 2 in skin-allografted rats[20]. More recently, NKT-01 was demonstrated to ameliorate ongoing rejection in rat allogeneic heart transplantation[21] and advancing graft-versus-host disease in mice[22]. In xenogeneic (hamster to rat) heart transplantation, this agent was found to be more effective than cyclosporine in prolonging graft survival[23]. While the mechanisms responsible for the immunosuppression elicited by NKT-01 remain to be unravelled, it is likely that these will be different from those of cyclosporine.

In conclusion, NKT-01 is beneficial as both a prophylactic and a curative therapy for lupus-like

lesions in MRL/1 mice. Although a mechanism linking the therapeutic activity of NKT-01 and NKT-01-induced immunosuppression in these autoimmune disease mice is not clear, both an impairment of B cell function and an enhancement of IL production by NKT-01 may in part be likely mechanisms for these effects. However, further studies on the mechanism of NKT-01 action in this model are needed.

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