

IN VIVO EFFECTS OF DEOXYSPERGUALIN (NKT-01) ON
LYMPHOCYTE ACTIVATION IN RESPONSE
TO ALLOANTIGENS

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We studied the effects of deoxyspergualin (NKT-01) on the events of lymphocyte activation *in vivo* by inoculating mice in the footpad with allogeneic spleen cells, and compared the effects with those of cyclosporin A (CyA). The administration of NKT-01 increased the numbers of cells recovered from the popliteal lymph node (PLN) 7 days after inoculation, but inhibited the proliferation of these cells in the presence of exogenous interleukin 2 (IL-2). NKT-01 enhanced IL-2 production, but suppressed the production of macrophage activating factor (MAF) in the mixed lymphocyte reaction between the PLN cells and allogeneic spleen cells treated with mitomycin C. CyA decreased the numbers of PLN cells little, and suppressed the response to exogenous IL-2 and the production of both IL-2 and MAF. Results with tumor cells used as allogeneic cells suggested that there was a close relationship between the suppression of MAF production by NKT-01 and its inhibition of allograft rejection. The findings showed that NKT-01 inhibited both the MAF production by and the response to IL-2 of PLN cells, and that these effects were involved in the suppression of allograft rejection by NKT-01.

NKT-01^{1,2)} is a derivative of the antitumor antibiotic spargualin³⁾. It is a potent immunosuppressive drug that suppresses both antibody formation and delayed-type hypersensitivity⁴⁾, prolongs allograft survival in several transplantation systems⁵⁾, and prevents the appearance of autoimmune diseases in mixed lymphocyte reaction (MLR)/MpJ-lpr/lpr and [NZB×NZW]F₁ mice^{6,7)}. The immunosuppressive mechanism of NKT-01 is not known. The production of interleukin 2 (IL-2) and IL-3 is enhanced by NKT-01 in some experimental systems^{8,9)}. Here, we studied the effects of NKT-01 on some of the immunological properties of cells from popliteal lymph node (PLN) in mice inoculated in the footpad with allogeneic cells, and compared the effects with those of cyclosporin A (CyA).

Materials and Methods

Animals

Female C57BL/6 mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Japan. Female BALB/c mice were purchased from Charles River Japan Inc. All mice used in the experiments were 6 to 8 weeks of age.

Drugs

NKT-01 was prepared by Takara Shuzo Co., Ltd.²⁾ CyA (Sandimmun) was purchased from the Sankyo Co., Ltd.

Transplantation of Allogeneic Spleen Cells

C57BL/6 (H-2^b) mice were inoculated in the footpad with 10⁷ spleen cells from BALB/c (H-2^d) mice. NKT-01 or CyA was injected intraperitoneally for 6 days at daily doses of 3.13 or 20 mg/kg, respectively, starting the day after the transplantation.

Cell Preparation

Seven days after transplantation, the PLN of the leg that had been injected was removed. The cells were suspended in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% fetal calf serum, benzylpenicillin (200 U/ml), kanamycin (60 µg/ml), L-glutamine (2 mM), and 2-mercaptoethanol (5 × 10⁻⁵ M). In some experiments, PLN cells were treated with anti-Thy-1.2 (First Chemicals Co., Ltd., Japan) for 45 minutes at 4°C, washed, and treated with guinea pig complement diluted 1:10 (Wako Pure Chemical Industries, Ltd., Japan) for 45 minutes at 37°C.

Response to Exogenous IL-2

PLN cells (2 × 10⁸) were cultured with or without mouse recombinant IL-2 (6 U, Nakarai Chemicals, Ltd., Japan) for 24 hours in a 96-well plate. During the final 6 hours of culture, 0.5 µCi of [³H]-thymidine ([³H]TdR; New England Nuclear Corp., U.S.A.) was added to each well and the incorporation of the label into the cultured cells was measured.

Preparation of Culture Supernatant Containing IL-2 and Macrophage Activating Factor (MAF)

The PLN cells (2 × 10⁸/ml) as responder cells were cultured with BALB/c spleen cells (2 × 10⁶/ml) that had been treated with 50 µg/ml mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Japan) as stimulator cells. After incubation for 24 hours, the culture supernatant of this mixed lymphocyte reaction was obtained.

IL-2 Assay

An IL-2-dependent cell line, CTLL-2 (kindly supplied by Dr. M. INABA of the Kansai Medical University), was cultured in a 96-well plate for 24 hours with 4 × 10³ cells/100 µl/well, with the addition of 100 µl of the supernatant of the MLR. We measured the incorporation of [³H]TdR (0.5 µCi/well) during the final 4 hours.

MAF Assay

The MAF activity was assayed in terms of the cytostatic activity of macrophages against mouse leukemia L5178Y cells. Peritoneal macrophages elicited with use of a thioglycolate medium were obtained from C57BL/6 mice, and 1 × 10⁴ cells in 180 µl of medium were mixed with 20 µl of the supernatant of the MLR in 96-well plates. After 24 hours of incubation, the cells were washed and 5 × 10³ L5178Y cells were added to each well as target cells. After incubation for 18 hours more, the cells were pulsed with 0.2 µCi of [³H]TdR for 6 hours. The cytostatic activity was calculated by this formula:

$$\% \text{ Cytostasis} = \left(1 - \frac{\text{cpm in culture with experimental macrophages}}{\text{cpm in culture of target cells only}} \right) \times 100.$$

Transplantation of Allogeneic Tumor Cells

C57BL/6 mice were inoculated in the footpad with 4 × 10⁶ cells of the mouse fibrosarcoma Meth A (H-2^d). NKT-01 was injected ip for 6 days at the daily dose of 3.13 mg/kg starting the day after tumor inoculation. The thickness of the footpad was measured with a thickness gauge every day or 2 for 20 days. Then, 2, 4, 6, 8, 11, or 20 days after the transplantation, the PLN of the leg that had received the tumor cells was removed. The MAF activity in the supernatant of the mixed lymphocyte-tumor reaction (MLTR) between the PLN cells and Meth A tumors treated with mitomycin C was assayed.

Statistical Analysis

Data were analyzed by Student's t-test.

Results

When spleen cells of BALB/c (H-2^d) mice were injected, the total number of cells recovered 7

days later from the PLN of the C57BL/6 (H-2^b) mice treated with saline was 6-fold the number recovered from untreated normal mice. When recipients were treated with NKT-01, the number of cells was greater than in these saline-treated controls. When CyA was used instead of NKT-01, the number of cells was slightly less than in the saline-treated controls (Table 1).

The proliferation of the PLN cells recovered in the presence of exogenous IL-2 was measured to evaluate their immunological functions (Table 2). In untreated normal mice, the cells had a little response to IL-2. However, cells from saline-treated control mice developed the response and proliferated in the presence of IL-2. When recipients were treated, this proliferation was inhibited by 73% compared to the saline-treated controls with NKT-01 and by 51% with CyA.

We next studied the effects of NKT-01 or CyA on the production of IL-2 and MAF by the PLN cells in the MLR between the cells and allogeneic spleen cells treated with mitomycin C. In culture supernatants of the PLN cells only, *i.e.* without stimulator cells, neither IL-2 nor MAF were detected (data not shown). NKT-01 enhanced IL-2 production by 27% compared to the saline-treated controls, and CyA suppressed it by 19% (Table 3). MAF production in the MLR was suppressed by both NKT-01 and CyA (Table 4), with the inhibition caused by NKT-01 being higher than that of CyA. The production of MAF was abolished by treatment of the PLN cells with anti-Thy-1.2 and complement (Table 5), which showed that the production of MAF in our system was mediated by T-cells.

NKT-01 was also tested for effects on the

Table 1. Effects of NKT-01 or CyA on numbers of cells recovered from the popliteal lymph nodes (PLN)^a.

	Millions of cells/PLN ^b (mean±SD)	T/C
Saline	5.46±0.55	100
NKT-01	8.04±1.56 ^c	147
CyA	5.20±1.29	92

^a C57BL/6 (H-2^b) mice were inoculated in the footpad with 10⁷ spleen cells from BALB/c (H-2^d) mice. NKT-01, CyA, or saline was injected intraperitoneally for 6 days at daily doses of 3.13 or 20 mg/kg, respectively, starting the day after the transplantation. Seven days after transplantation (1 day after the last injection), the PLN of the leg that had received the spleen cells was removed and the cells were counted. Each value is the mean from five experiments, each with 5 mice.

^b The number of cells recovered from untreated normal mice was 9.1 × 10⁵ cells/PLN, which was the mean from 11 mice.

^c P<0.01.

Table 2. Effects of NKT-01 or CyA on response to exogenous IL-2 of the PLN cells.

	IL-2 (U/ml)	[³ H]TdR* incorporation (cpm±SD)	Stimulation index ^b	Inhibition ^c (%)
Saline	—	434±98	7.8	0
	30	3,270±247		
NKT-01	—	315±44	2.1	73
	30	676±79		
CyA	—	341±137	3.8	51
	30	1,288±210		

^a PLN cells were obtained from C57BL/6 mice treated as described in the footnote to Table 1 and cultured for 24 hours with or without mouse recombinant IL-2. In untreated normal mice, cpm in culture without IL-2 was 155±27, and cpm with 30 U/ml IL-2 was 419±56. Each value is the mean cpm from triplicate counts.

^b Stimulation index = $\frac{\text{cpm in culture with 30 U/ml IL-2}}{\text{cpm in culture without IL-2}}$.

^c Inhibition (%) = $\left(1 - \frac{\text{stimulation index with experimental mice}}{\text{stimulation index with saline-treated control mice}}\right) \times 100$.

Table 3. Effects of NKT-01 or CyA on IL-2 activity in culture supernatant of the mixed lymphocyte reaction.

	^3H TdR incorporation ^a (cpm \pm SD)	T/C
Saline	4,775 \pm 396	100
NKT-01	6,073 \pm 232 ^b	127
CyA	3,870 \pm 186 ^b	81

^a PLN cells were obtained from C57BL/6 mice treated as described in the footnote to Table 1 and cultured for 24 hours with BALB/c spleen cells that had been treated with mitomycin C. The IL-2 activity of the supernatant against the IL-2-dependent cell line CTLL-2 was assayed. Each value is the mean cpm from triplicate counts.

^b $P < 0.01$.

Table 5. Effects of treatment with anti-Thy-1.2 and complement of PLN cells on the production of MAF.

Treatment	MAF activity ^a (% cytoxicity)
—	33.7 \pm 2.8
Anti-Thy-1.2	23.4 \pm 1.7
Complement	31.0 \pm 4.9
Anti-Thy-1.2+complement	2.7 \pm 3.6 ^b

^a PLN cells were obtained from saline-treated control mice and treated with anti-Thy-1.2, complement, or both. After being washed, the cells were cultured for 24 hours with allogeneic spleen cells that had been treated with mitomycin C. The MAF activity of the supernatant was assayed (E:T=2:1). Each value is the mean percentage of cytoxicity from triplicate assays.

^b $P < 0.005$.

allogeneic transplantation of tumor cells. When allogeneic tumor cells were used as allografts, we estimated the acceptance or rejection of allografts by measuring the thickness of the footpad. In saline-treated control mice, Meth A (H-2^d) tumors grew steadily for the first 6 days, and then rapidly regressed as the immunological rejection mechanism began to act (Fig. 1). NKT-01 delayed the rejection of tumor allografts. When the daily dose of NKT-01 was 3.13 mg/kg, rejection started on day 12, 6 days after the last injection. The changes in MAF activity in the supernatant of the MLTR resembled those for footpad thickness (Fig. 2). In saline-treated control mice, MAF production started on day 4 after transplantation, with the peak of the activity at day 6,

Table 4. Effects of NKT-01 or CyA on MAF activity in culture supernatant of a mixed lymphocyte reaction.

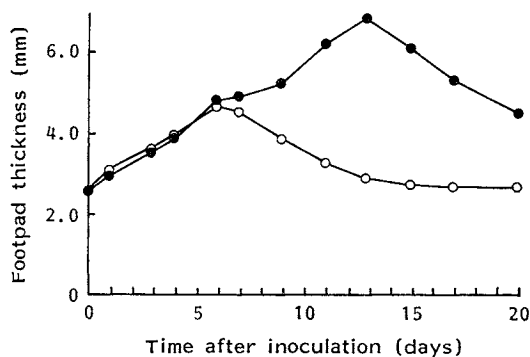
	^3H TdR incorporation ^a (cpm \pm SD)	MAF activity (% cytoxicity)
Saline	9,390 \pm 92	59.5
NKT-01	21,301 \pm 464 ^b	9.1
CyA	17,185 \pm 185 ^b	26.7

^a PLN cells were obtained from C57BL/6 mice treated as described in the footnote to Table 1 and cultured for 24 hours with BALB/c spleen cells that had been treated with mitomycin C. The MAF activity of the supernatant was assayed in terms of the cytoxic activity of macrophages against mouse leukemia L5178Y cells (E:T=2:1). The counts per minute of L5178Y cells were 23,414 \pm 802 without macrophages. Each value is the mean cpm from triplicate counts.

^b $P < 0.01$.

Fig. 1. Effects of NKT-01 on the growth of allogeneically transplanted tumor cells.

○ Saline, ● NKT-01.



C57BL/6 mice were inoculated in the footpad with 4×10^6 Meth A tumor cells. NKT-01 was injected intraperitoneally for 6 days at the daily dose of 3.13 mg/kg, starting the day after tumor transplantation. The thickness of the footpad was measured with a thickness gauge every day or 2 for 20 days. Each point is the mean of values from four animals.

and the production dropped to the level in the untreated normal mice on day 11. NKT-01 inhibited MAF production during its administration. In mice treated with NKT-01, MAF production started on day 11 (5 days after the last injection), the day before the start of the rejection of allogeneic tumors.

Discussion

We found some immunosuppressive modes of NKT-01 using PLN cells from mice that were inoculated in the footpad with allogeneic cells.

First, NKT-01 significantly inhibited the response to exogenous IL-2 by the PLN cells, but IL-2 production in the MLR between the cells and allogeneic spleen cells was enhanced in mice treated with NKT-01. Both response and production were inhibited by CyA. NKT-01 had a different mechanism from CyA. NKT-01 enhanced IL-2 production, so the inhibition of the response to IL-2 was not caused by decreased IL-2 production. We speculate that NKT-01 decreases the number of IL-2 receptors, or else impairs the function of the IL-2 receptor directly, so that the T-cells that participate in allograft rejection are not activated.

Second, NKT-01 suppressed MAF production by the PLN cells in the MLR. In the experiment with allogeneic tumor cells used as allografts, there seemed to be a close relationship between the suppression of MAF production and the inhibition of allograft rejection. DICKNEITE *et al.*⁵⁾ observed that NKT-01 suppresses the secretion of both hydrolytic enzymes and IL-1 and also suppresses the chemiluminescence reaction in monocytes/macrophages during rat skin transplantation. However, MASUDA *et al.*⁴⁾ have reported that immunosuppressive NKT-01 does not reduce functions of zymosan-activated macrophages such as the release of lysosomal enzymes and the production of superoxide anions, so the effects of NKT-01 on the functions of macrophages are uncertain. We could not assay the functions of macrophages in the PLN because there were too few. The suppression of MAF production by NKT-01 would inhibit macrophage activation, and macrophages act in allograft rejection¹⁰⁻¹²⁾. Thus, the suppression of MAF production by NKT-01 participates in the suppression of allograft rejection.

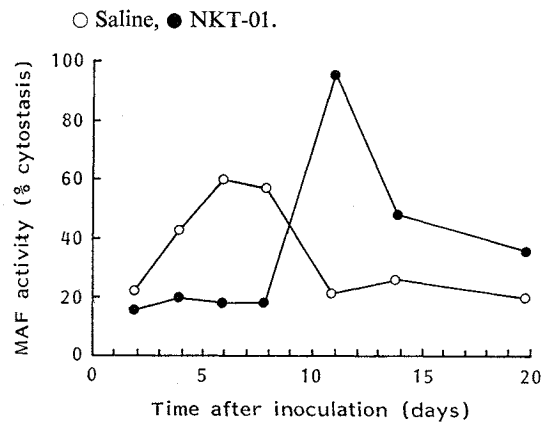
NKT-01 is a new kind of immunosuppressive drug. It affected some functions mediated by T-cells, so that it inhibited the response to IL-2 and suppressed MAF production. Also, NKT-01 increased the numbers of cells recovered from the PLN 7 days after inoculation. The distribution of lymphocyte subsets in the PLN may be changed greatly by NKT-01. More study is needed to understand the mechanism of NKT-01 in the response of hosts to allografts.

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Fig. 2. Effects of NKT-01 on MAF production by the PLN cells from mice bearing allogeneic tumor cells.



Mice received Meth A tumor, and 2, 4, 6, 8, 11, 14, or 20 days later, the PLN of the mice was removed. The cells were cultured for 24 hours with Meth A tumor cells treated with mitomycin C. The MAF activity in the supernatant was assayed in terms of the cytostatic activity of macrophages against L5178Y cells (E:T=4:1). Each value is the mean percentage of cytostasis from triplicate assays.

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