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In vivo Antitumor Mechanism of Natural Human Tumor Necrosis Factor Involving a T Cell-mediated Immunological Route

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We have investigated the *in vivo* antitumor mechanism of natural human tumor necrosis factor (n-TNF) isolated from a culture of human leukemic B cell line (BALL-1), especially its action as an immunomodulator, and found that the *in vivo* antitumor effect of n-TNF on Meth A sarcoma implanted in BALB/c mice pretreated with monoclonal antibody against T cell-specific surface antigen (Thy-1) was significantly diminished. Furthermore, when BALB/c mice were treated with T cell subset-specific monoclonal antibodies, anti-L3T4 or anti-Lyt-2.2, the antitumor effect of n-TNF on Meth A sarcoma was significantly reduced. Therefore, it was suggested that the *in vivo* antitumor mechanism of n-TNF might involve a T cell-mediated immunological route.

Key words: Tumor necrosis factor — *In vivo* antitumor mechanism — T cell subset — Monoclonal antibody

Tumor necrosis factor (TNF) was firstly reported as a macrophage-derived serum factor that caused hemorrhagic necrosis of tumors,[1] and it has been reported by many investigators to exhibit antitumor activities against both murine tumors transplanted in syngeneic mice,[2-5] and human tumors transplanted in nude mice.[2,5] It is now well-known that in addition to its direct *in vitro* antiproliferative and *in vivo* antitumor activities, TNF has a wide range of biological activities both *in vitro*[6-12] and *in vivo*.[13-16] However the exact mechanism mediating the *in vivo* antitumor effect of TNF has not yet been clarified.

We have reported that purified natural human TNF (n-TNF) isolated from the culture of human leukemic B cell line (BALL-1)[17] inhibits neoplastic cell proliferation both *in vitro* and *in vivo*, and acts synergistically with human interferon-α.[18] In the present study, we have investigated the *in vivo* antitumor mechanism of n-TNF, especially its action as an immunomodulator.

n-TNF used in this study was purified as described in detail elsewhere[18] and had a specific activity of over 1.5×10^6 JRU (Japan Reference Unit[19])/mg of protein.

Meth A cells (2×10^5) were implanted intradermally in the backs of 5- to 6-week-old female BALB/c and BALB/c *nu-nu* mice (Clea Japan Inc.) after which mice with tumor volumes of 100–300 mm^3 were selected and injected intratumorally with 5.7×10^3 JRU/mouse of n-TNF. Hemorrhagic necrosis at the tumor site on day 1 after the injection and significant regression of tumor growth were observed in BALB/c mice, with 5 out of 6 animals showing complete regression 2 weeks after the injection. On the other hand, such effects could be hardly seen in BALB/c *nu-nu* mice and the antitumor effect of n-TNF was greatly reduced (Fig. 1), as Haranaka *et al.*[5] had found using murine TNF.

Such an experimental result with nude mice lacking T cell-specific immunity drew our attention, leading us to investigate further the antitumor effect of n-TNF as a T cell modulator. Meth A cells (2×10^5) were implanted in BALB/c mice, after which the animals were administered intravenously with 10 μl/mouse of anti-mouse Thy-1.2 monoclonal antibody (anti-Thy-1 antibody) once a day for 8 days. When these anti-Thy-1 antibody-treated mice were injected with 6×10^3 JRU/mouse of n-TNF, it was observed that the antitumor effect was reduced compared with that in the animals which did not receive the pretreatment with the antibodies (Fig. 2). This indicates that n-TNF has an indirect *in vivo* antitumor effect as a T cell modulator in contrast to its known direct *in vitro*[20-23] tumor-regressing effect.

In order to determine the T cell subsets which mediate this antitumor effect of n-TNF, we conducted a detailed examination using monoclonal antibodies specific to T cell subsets and flow cytometry. Meth A cells (2×10^5)

were implanted in BALB/c mice, after which the animals were treated 3 times with monoclonal antibodies recognizing surface antigens of helper T cells or killer T cells (anti-L3T4 antibody or anti-Lyt-2.2 antibody) on the day of implantation and on days 7 and 14 after implantation. On the 7th day after implantation 6×10^3 JRU/mouse of n-TNF was injected, and the antitumor effect of n-TNF was compared with that in mice which did not

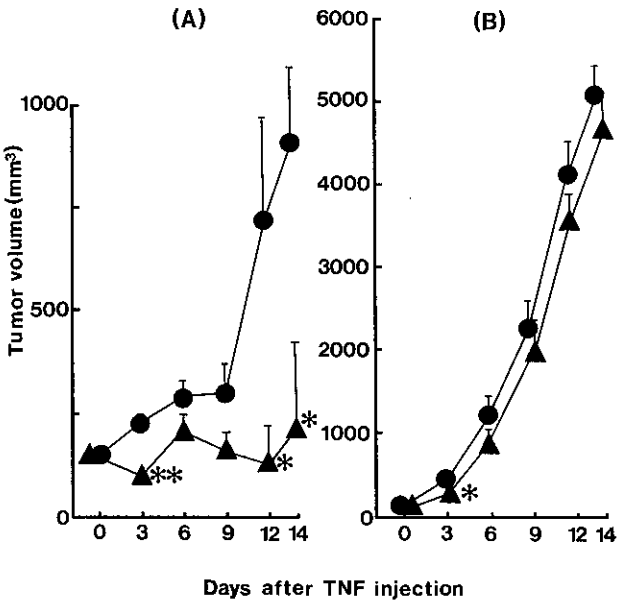

Fig. 1. Comparison of antitumor effect of n-TNF on Meth A cells transplanted in BALB/c mice and BALB/c *nu-nu* mice. Meth A cells (2×10^5) were respectively implanted in six 5- to 6-week-old BALB/c mice (A) and in six 5- to 6-week-old BALB/c *nu-nu* mice (B). At 7 days after implantation, the animals received an intratumoral injection of 5.7×10^3 JRU/mouse of n-TNF (△) or saline (○). After the injection, the diameters of the tumors were measured using a caliper and tumor volume was calculated in mm^3 as: $1/2\times(\text{width})^2\times(\text{length})$. The results show the mean±SE, and * and ** indicate significant differences from control ($P<0.05$ and $P<0.01$, respectively).

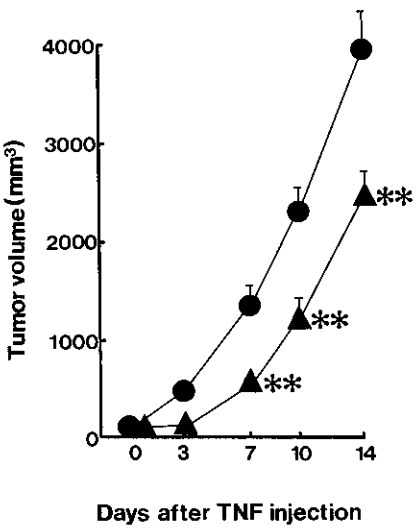

Fig. 2. Antitumor effect of n-TNF on anti-Thy-1 antibody-treated BALB/c mice. Meth A cells (2×10^5) were implanted in six 5- to 6-week-old BALB/c mice. The animals were then intravenously administered with 10 μl/mouse of anti-Thy-1.2 monoclonal antibody (Cedarlane Laboratories Ltd.) once a day for 8 days starting from the day of implantation, and injected intratumorally with 6×10^3 JRU/mouse of n-TNF (△) or saline (○) on day 7 after implantation. The tumor volumes were measured periodically in the same manner as described in the legend to Fig. 1. The results show the mean±SE and ** indicates a significant difference from the control ($P<0.01$).

Table I. Influence of Anti-T Cell Subset Antibody on the Antitumor Effect of n-TNF

Group	Dose (JRU/mouse)	Antibody (μl/mouse)	Tumor volume (mm^3)
Control	——	——	1719±206
n-TNF	6×10^3	——	170±59
Anti-L3T4 antibody	——	50×3	1545±175
n-TNF+anti-L3T4 antibody	6×10^3	50×3	799±239
Anti-Lyt-2.2 antibody	——	30×3	2248±148
n-TNF+anti-Lyt-2.2 antibody	6×10^3	30×3	1640±170

Meth A cells were implanted in BABL/c mice in the same manner as described in the legend to Fig. 2 and the animals were administered intravenously with 50 μl/mouse of anti-L3T4 monoclonal antibody (Sera Lab. Ltd.) or 30 μl/mouse of anti-Lyt-2.2 monoclonal antibody (Meiji Institute of Health Science) on the day of implantation and on days 7 and 14 after implantation. On day 7 after implantation, 6×10^3 JRU/mouse of n-TNF was injected intravenously into mice pretreated with the respective antibody and the tumor volume (mean±SE of 5 mice) 10 days after this injection is shown in the table.

Table II. Effect of Anti-T Cell Subset Antibody on Lymphocyte Population

Source of lymphocytes	Antibody	Cell number (% of control)		
		Thy-1$^+$	L3T4$^+$	Lyt-2.2$^+$
Lymph node	——	100	100	100
	Anti-Thy-1	2.7	5.3	0.4
	Anti-L3T4	36	6.8	96
	Anti-Lyt-2.2	74	101	0
Peripheral blood	——	100	100	100
	Anti-Thy-1	4.5	3.9	0.9
	Anti-L3T4	50	0.4	144
	Anti-Lyt-2.2	68	94	0

BABL/c mice pretreated with anti-Thy-1 antibody, anti-L3T4 antibody or anti-Lyt-2.2 antibody in the same manner as described in Fig. 2 and Table I were killed on the 7th day and the lymphocytes from their lymph nodes and peripheral blood were obtained using conventional methods. Lymphocytes (2.5×10^5~5×10^5 cells) were double-stained with T cell subset-specific antibodies labelled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)[24] (kindly provided by Prof. Ko Okumura, Juntendo University School of Medicine, Tokyo). The subsets in which these lymphocytes were involved were determined by using a flow cytometer (FACScan, Becton Dickinson). The number of lymphocytes in each subset in the control group was taken as 100% and results are the mean values of 2 mice.

receive the pretreatment with the antibodies. The antitumor effect of n-TNF in mice pretreated with anti-L3T4 antibody or anti-Lyt-2.2 antibody was significantly reduced, being similar to that when the mice were pretreated with anti-Thy-1 antibody (Table I). Furthermore, no case of complete regression was observed. Also, it was observed by flow cytometry that pretreatment with the respective monoclonal antibody specifically decreased the number of the corresponding T cell subset in the lymph nodes and peripheral blood (Table II), while the amount of the surface H-2 antigens on Meth A cells was unaffected even after treatment with n-TNF up to 3×10^3 JRU/ml *in vitro* (data not shown).

These results suggest that the *in vivo* antitumor effect of n-TNF mainly involves L3T4 antigen-positive cells and Lyt-2.2 antigen-positive cells. Also, as the antitumor effect of n-TNF is diminished by treatment with anti-L3T4 antibody and L3T4 antigen-positive cells are thought to have no direct tumor-killing activity, it can be deduced that n-TNF first activates the helper T cells, L3T4 antigen-positive cells, and then the stimulated helper T cells activate the cytotoxic T lymphocytes (CTL), Lyt-2.2 antigen-positive cells, to bring about tumor regression.

Thus, our hypothesis regarding the antitumor mechanism of n-TNF is as follows. i) n-TNF activates L3T4 antigen-positive cells (helper T cells), directly or indirectly. ii) Activated L3T4 antigen-positive cells activate Lyt-2.2 antigen-positive cells (CTL). iii) Activated CTL directly destroy tumor cells. Furthermore, although we have focused our attention on Meth A cells in this study, as the antitumor effect of n-TNF was also found to be greatly diminished in studies on colon 26 adenocarcinoma-bearing mice pretreated with anti-Thy-1 antibodies (data not shown), it can be deduced that n-TNF's action as a T cell modulator may be a very general one.

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