Anti-Cancer Drugs 1998, 9, pp. 273–282

Research paper

ONO-4007 induces specific anti-tumor immunity mediated by tumor necrosis factor-α

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We investigated the therapeutic effects of ONO-4007, a novel synthetic lipid A derivative with low toxic activities, on transplanted hepatocellular carcinoma KDH-8 in WKAH rats. ONO-4007 brought about complete cures in about 60% of rats bearing tumor necrosis factor (TNF)-α-sensitive KDH-8 cells, whereas no complete cure was observed in rats bearing cKDH-8/11 which is identical to KDH-8 but a TNF-α-resistant cell line, KMT-17 and KEG-1. Then we examined the influence of rabbit anti-TNF-α antibody on the therapeutic effects of ONO-4007 against the TNF-α-sensitive KDH-8. The concomitant administration of the rabbit anti-TNF-α antibody completely abrogated the therapeutic effects of ONO-4007. On the other hand, rechallenged tumor cells of both KDH-8 and cKDH-8/11 were completely rejected in the rats cured of KDH-8 tumor, although no rejection of KEG-1 was observed. Moreover, Winn assay, i.e. the tumor cell neutralizing assay, indicated that $CD4^+$ T cells were involved in the antigen-specific transplantation resistance. These findings suggest that antigen-specific T cell responses are involved in the complete cure of tumors after the treatment with ONO-4007, although its therapeutic effect is initiated by TNF-α. [© 1998 Rapid Science Ltd.]

Key words: CD4 T cell, lipid A, transplantation resistance, tumor necrosis factor-α.

Introduction

Although lipopolysaccharide (LPS) has potent anti-tumor activities, it has not been used clinically yet because of its severe adverse effects.[1,2] Recently, several synthetic lipid A derivatives with less severe adverse effects have been developed for cancer treatment.[3–10] We have recently reported that ONO-4007, a novel lipid A derivative with low toxicity of less than 1/1000 that of natural LPS derived from *Escherichia coli*, induced the production of tumor necrosis factor (TNF)-α selectively in the tumor tissues.[11–21] We have also reported that *in vivo* treatment with i.v. administration of ONO-4007 was well tolerated by rats and that ONO-4007 cured half of the rats implanted with KDH-8 cells that were sensitive to TNF-α.[19–21] On the other hand, *in vitro* treatment with TNF-α inhibited the proliferation of KDH-8 cells by only 20–50%, compared with the *in vivo* effect of ONO-4007.

These data prompted us to speculate that effectors other than TNF-α might be involved in the rejection of KDH-8 cells *in vivo*. As it has been reported that lipid A stimulates the production of various cytokines including IL-1, IL-6, TNF-α and granulocyte macrophage colony stimulating factor,[22–30] these monokines may turn on the cytokine production cascade and bring about the induction of T cell immune response.[19] Alternatively, ONO-4007 may induce the activation of antigen-presenting cells, resulting in the induction of T cell immune response. Therefore, we examined first whether anti-TNF-α antibody could abrogate the anti-tumor effects of ONO-4007 to determine the role of TNF-α in the eradication of KDH-8 cells *in vivo* by administration of ONO-4007. Then we examined whether transplantation resistance could be induced against the rechallenged KDH-8 cells in the rats cured with ONO-4007. Finally, we determined the phenotype of effector cells involved in the transplantation resistance in the rats cured with ONO-4007.

Materials and methods

Animals and cell lines

Female Wister King Aptekman/Hok (WKAH) rats, 8–12 weeks old, were supplied by the Experimental Animal

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture, and the Ministry of Health and Welfare, Japan. We wish to acknowledge ONO Pharmaceutical for kindly providing ONO-4007.

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Institute of the Hokkaido University School of Medicine (Sapporo, Japan). KDH-8 is a transplantable rat hepatocellular carcinoma cell line established from a tumor induced by 3'-methyl-4-dimethylaminoazobenzene in a WKAH rat. KMT-17 is a transplantable rat fibrosarcoma cell line established from a tumor induced by methylcholanthrene in a WKAH rat. These two cell lines were maintained in ascites form by i.p. passage every 5–7 days. cKDH-8/11 is a subclone of KDH-8 for maintaining in culture form. A3 is also a subclone of KMT-17 for maintaining in culture form. Mean survival times of the rats implanted with 1×10^5 cells of KDH-8, cKDH-8/11 and KMT-17 are about 50, 50 and 25 days, respectively, after implantation. KEG-1 is a transplantable rat glioblastoma cell line established from a tumor induced by 1-ethyl-1-nitrosourea in a WKAH rat. This cell line was maintained in culture form. Mean survival time of the rats implanted with 1×10^5 cells of KEG-1 is about 65 days after implantation.

Reagents

ONO-4007, sodium 2-deoxy-2-[3*S*-(9-phenylnonanoyloxy) tetradecanoyl]-amino-3-*O*-(9-phenylnonanonyl)-D-glucopyranose 4-sulfate, was kindly provided by ONO Pharmaceutical (Osaka, Japan). Figure 1 shows its chemical structure. For *in vivo* use ONO-4007 was dissolved in 50% ethanol at 50 mg/ml and then diluted with distilled water to appropriate concentrations. TNF-α was purchased from Genzyme (Cambridge, MA).

In vivo antitumor effects

On day 0, 1×10^5 KDH-8, cKDH-8/11, KMT-17 and KEG-1 cells were implanted s.c., respectively, in WKAH rats. ONO-4007 or phosphate-buffered saline (PBS) was administered i.v. on days 7, 14, 21 and 28 to KDH-8- or cKDH-8/11-bearing rats, on days 5, 10 and 15 to KMT-17-bearing rats, and on days 7, 14, 21, 28 and 35 to KEG-1-bearing rats. Tumor size was measured every 3 or 4 days. The tumor-bearing rats were observed up to 90 days after tumor implantation when no tumor was detected in any of the surviving rats.

Rabbit anti-TNF-α antibody

Rabbit anti-TNF-α antibody was made by immunizing rabbit with rat recombinant TNF-α (Pepro Tech, Rocky Hill, NJ). The concentration of the obtained antibody was 152 mg protein/ml; 1 ml of this antibody had a neutralizing activity of 2 mg TNF-α. Samples of 6.08 mg protein of the antibody were administered i.v. four times 30 min prior to administration of ONO-4007 on days 7, 14, 21 and 28.

Preparation of tissue homogenate

On day 0, 1×10^5 KDH-8 cells were implanted s.c. in WKAH rats. Samples of 3 mg/kg of ONO-4007 and the antibody were administered i.v. four times on days 7, 14, 21 and 28 to KDH-8-bearing rats. The rats were killed 90 min after the administration of ONO-4007 on day 28. The tumor tissues were resected and homogenized in 1 ml of RPMI 1640 medium supplemented with 10% FBS per 100 mg tumor tissue. After centrifugation at 20 000 *g* for 60 min, they were passed through 0.45 μm pore size filters and used for assay of TNF-α, interleukin (IL)-1β and interferon (IFN)-γ assay. The sera were pooled and stored at −20°C until use.

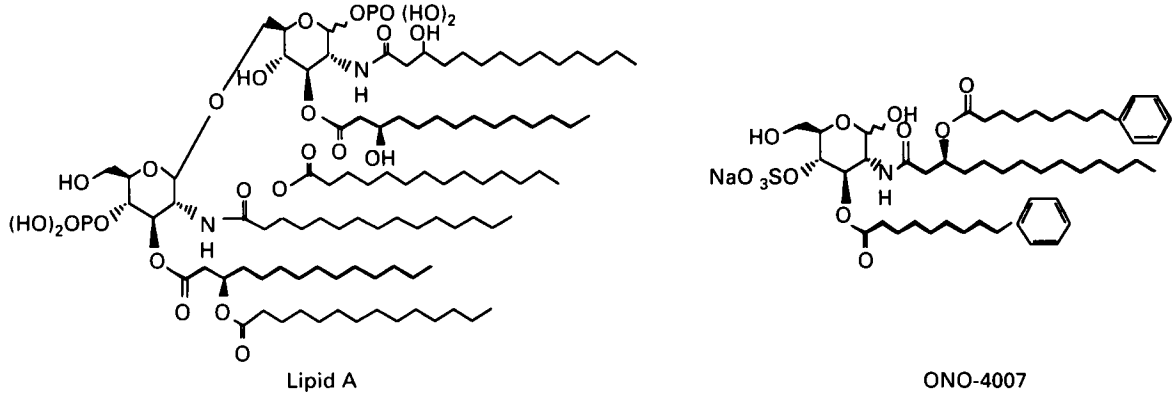

Figure 1. Chemical structures of Lipid A and its derivative, ONO-4007.

Cytokine concentration

Concentrations of TNF-α, IL-1β and IFN-γ were measured with ELISA kits (BioSource International, Camarillo, CA).

Immunization with tumor cells and tumor cell challenge

Tumor cells (1×10^7) were incubated with mitomycin (MMC) (50 μg/ml) in 1 ml RPMI medium supplemented with 10% FBS at 37°C for 1 h. After washing with phosphate-buffered saline (PBS), 1×10^6 tumor cells were implanted s.c. in WKAH rats three times on days 0, 7 and 14. On day 21, 1×10^5 viable tumor cells were implanted s.c.

Preparation of spleen cells

Spleens were removed aseptically from the cured rats and normal rats. Cell suspension was prepared by disrupting spleens with PBS in a loose fitting glass homogenizer and by passing the cells through four layers of sterilized gauze. After red blood cells were lysed with Tris-buffered ammonium chloride, the cell suspension was washed three times by centrifugation in cold RPMI 1640 medium supplemented with 10% FBS.

In vivo tumor neutralization assay (Winn assay)

The Winn assay[31] was performed according to the previously described method.[32] Briefly, spleen cells (1×10^7 and 1×10^8/ml) were mixed with tumor cells (1×10^6/ml, each) at an equal volume and then the mixture (0.2 ml) was implanted s.c. into syngeneic WKAH rats irradiated with 300 rad 1 day before the implantation. Inhibition of tumor growth was evaluated by the following formula, where A stands for the mean tumor weight in the rats implanted with tumor cells alone and B stands for the mean tumor weight in the rats implanted with both the mixture of spleen cells and tumor cells: % inhibition of tumor growth=$(1-B/A) \times 100$.

Preparation of subpopulation

$CD4^+$ cells were prepared from whole spleen cells by incubation with anti-CD8 mAb OX-6 (PharMingen, San Diego, CA) and anti-granulocytes and macrophages mAb R2-1A6a (Seikagaku, Tokyo, Japan), which was followed by incubation with complement CL-3061 (Cedarlane, Hornby, Ontario, Canada). $CD8^+$ cells were prepared from whole spleen cells by incubation with anti-CD4 mAb OX-35 (PharMingen) and anti-granulocytes and macrophages mAb R2-1A6a, which was followed by incubation with complement. Macrophages were prepared from spleen cells adherent to plastic culture dishes by incubation with anti-CD3 mAb G4.18 (PharMingen) which was followed by complement CL-3061 lysis. These cell populations were enriched, which was confirmed by FACScan (Becton Dickinson, Mountain View, CA).

Statistical analysis

Arithmetic means were calculated for each experiment group and tested for statistically significant differences by Student's *t*-test. Survival ratios were analyzed by contingency table analysis.

Results

Therapeutic effects of ONO-4007 on hepatocellular carcinoma KDH-8 in WKAH rats

Figure 2 shows the individual growth curves of KDH-8 tumors in WKAH rats treated with 3 mg/kg of ONO-4007 and PBS-treated rats. In PBS-treated rats, the average diameter of the tumors reached more than 40 mm. About 50% of ONO-4007-treated rats survived up to 90 days, whereas no untreated rats survived more than 45 days. Table 1 shows a summary of five different experiments. Although all untreated rats died, 54.1% of ONO-4007-treated rats survived. Mean survival times of dead untreated rats and ONO-4007-treated rats were 47.7 ± 6.7 and 53.8 ± 9.7 days, respectively.

Effects of ONO-4007 on the *in vivo* growth of the other cell lines in WKAH rats

Table 2 shows the effects of ONO-4007 on the growth of cKDH-8/11 cells which had been maintained in culture (KDH-8 cells had been maintained in ascites form), and those of fibrosarcoma KMT-17 cells and glioblastoma KEG-1 cells. Therapeutic effects against these cells were not observed. Then we examined the characteristics of these cells from the viewpoint of TNF-α sensitivity because ONO-4007 produced a large

amount of TNF-α only in tumor tissues. All these cell lines were resistant to TNF-α-mediated cytotoxicity (as shown in Table 3).

Abrogative effects of anti-TNF-α antibody on the *in vivo* effects of ONO-4007

Figure 3 shows the individual growth curves of KDH-8 tumors in WKAH rats treated with 3 mg/kg ONO-4007, 3 mg/kg ONO-4007 plus rabbit anti-TNF-α antibody, and 3 mg/kg of ONO-4007 plus preimmunized control rabbit Ig. Although the control Ig had no effect, anti-TNF-α antibody completely abrogated the therapeutic effects of ONO-4007.

Effects of anti-TNF-α antibody on the production of TNF-α and IL-1β in the tumor tissues

Table 4 shows the effects of anti-TNF-α antibody on the production of TNF-α and IL-1β in the tumor tissues. In the tumor tissue extracts of ONO-4007-treated rats, mean ± SD of TNF-α concentration was 101.1 ± 23.9 ng/ml, whereas that of those treated with ONO-

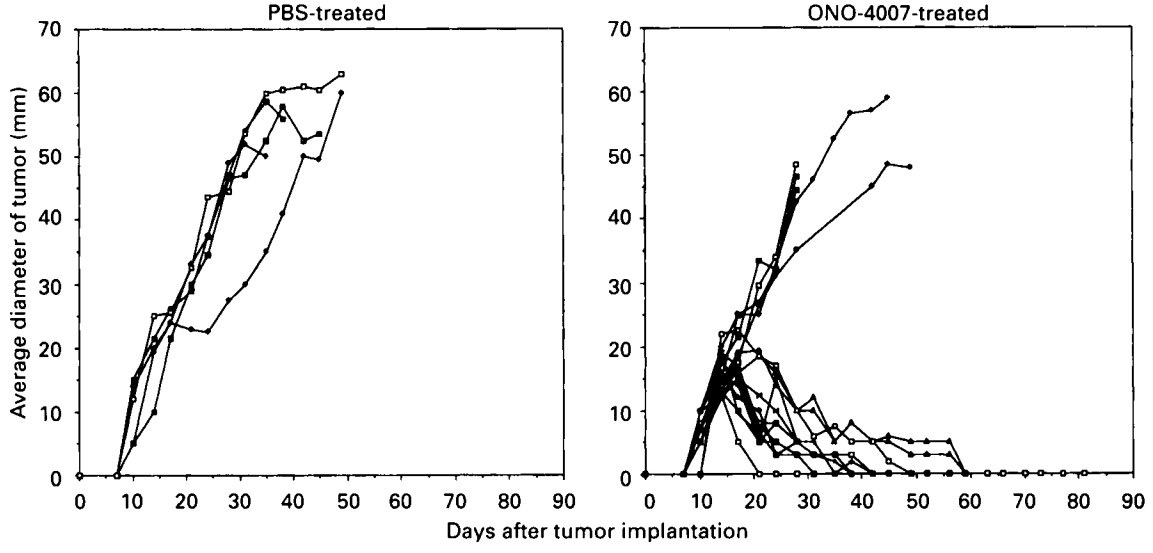

Figure 2. Individual growth curves of rat hepatocellular carcinoma KDH-8 in WKAH rats treated with PBS or ONO-4007. KDH-8 cells (1×10^5) were implanted s.c. in WKAH rats on day 0. ONO-4007 (3.0 mg/kg) or PBS was administered i.v. four times on days 7, 14, 21 and 28. The tumor size was measured every 3 or 4 days. Tumor-bearing rats were observed until their death. One representative of five independent experiments is shown.

Table 1. Therapeutic effects of ONO-4007 on KDH-8 tumors in WKAH rats

Experiment	Cured/treated (MST ± SD)[a]			
	PBS		ONO-4007	
1	0/5	(45.8 ± 6.5)	13/19	(60.3 ± 20.6)
2	0/3	(58.7 ± 11.0)	13/20	(61.4 ± 14.0)
3	0/3	(44.6 ± 0.6)	10/16	(50.0 ± 11.6)
4	0/10	(46.5 ± 5.3)	12/29	(52.2 ± 6.3)
5	0/5	(47.4 ± 2.6)	11/25	(52.1 ± 4.7)
Total	0/26	(47.7 ± 6.7)[b]	59/109	(53.8 ± 9.7)[b]

KDH-8 cells (1×10^5) were implanted s.c. in WKAH rats on day 0. ONO-4007 (3.0 mg kg) or PBS was administered i.v. four times on days 7, 14, 21 and 28.
[a]Mean survival time of dead rats ± SD.
[b]Statistically significant ($p < 0.01$).

Table 2. Therapeutic effects of ONO-4007 on various tumors in WKAH rats

Tumor cells[a] implanted	Cured/treated (MST ± SD)[b]			
	PBS		ONO-4007[c]	
cKDH-8/11	0/5	(49.6 ± 7.0)	0/15	(56.3 ± 10.9)
KMT-17	0/5	(15.6 ± 2.2)	0/11	(16.7 ± 5.3)
KEG-1	0/3	(73.3 ± 9.1)	0/5	(70.4 ± 6.2)

[a]Tumor cells (1×10^5) were implanted s.c. in WKAH rats on day 0.
[b]Mean survival time of dead rats ± SD.
[c]ONO-4007 (3.0 mg kg) or PBS was administered i.v. four times on days 7, 14, 21 and 28 to cKDH-8/11-bearing rats, three times on days 5, 10 and 15 to KMT-17-bearing rats, and five times on days 7, 14, 21, 28 and 35 to KEG-1-bearing rats.

Table 3. The effects of natural human TNF-α on *in vitro* growth of KDH-8, cKDH-8/11, A-3 and KEG-1 cells

Concentration of TNF-α (U/ml)	Inhibition (%)			
	KDH-8	cKDH-8/11	A-3	KEG-1
1000.0	20.9	2.2	1.8	1.4
500.0	20.0	2.7	2.5	0.0
250.0	18.0	2.1	2.3	−0.9
125.0	15.0	2.1	1.5	−0.9
62.5	11.6	2.8	3.5	−0.3
31.3	6.9	3.8	5.0	0.8
15.6	4.5	2.4	3.5	0.8
7.8	3.5	3.1	2.2	0.9
3.9	2.0	2.6	1.3	0.7
2.0	1.7	2.2	0.6	−1.3
1.0	1.4	1.0	0.8	−0.8

These data were obtained by a colorimetric crystal violet stain assay. Cells (1×10^4) were plated in 100 μl of medium in 96-well flat-bottomed microplates and incubated for 24 h, then cells were treated with recombinant human TNF-α for 48 h. Percent inhibition $=(OD_{med} - OD_{TNF})/OD_{med} \times 100$. OD_{med}; OD of wells containing the cells and medium. OD_{TNF}; OD of wells containing the cells and TNF-α at the concentration indicated above.

4007 plus anti-TNF-α antibody was 10 ng/ml. Anti-TNF-α antibody slightly but not significantly decreased the production of IL-1β.

Transplantation resistance in the rats cured with ONO-4007

Table 5 shows the results of two independent experiments of rechallenging KDH-8 cells. Rechallenged KDH-8 cells, even large number of KDH-8 cells, were rejected in all the cured rats. Furthermore, as shown in Table 6, cKDH-8/11 cells which were resistant to cytotoxicity of TNF-α were also rejected in the cured rats. However, KEG-1 cells which were also resistant to cytotoxic effects of TNF-α grew lethally in the cured rats. These results indicated that the rejection of rechallenged KDH-8 cells was not mediated by TNF-α. As immunization with MMC-treated-KDH-8 cells failed to induce the KDH-8-specific immune response as shown in Figure 4, KDH-8 cells must have been low-immunogenic tumor cells.

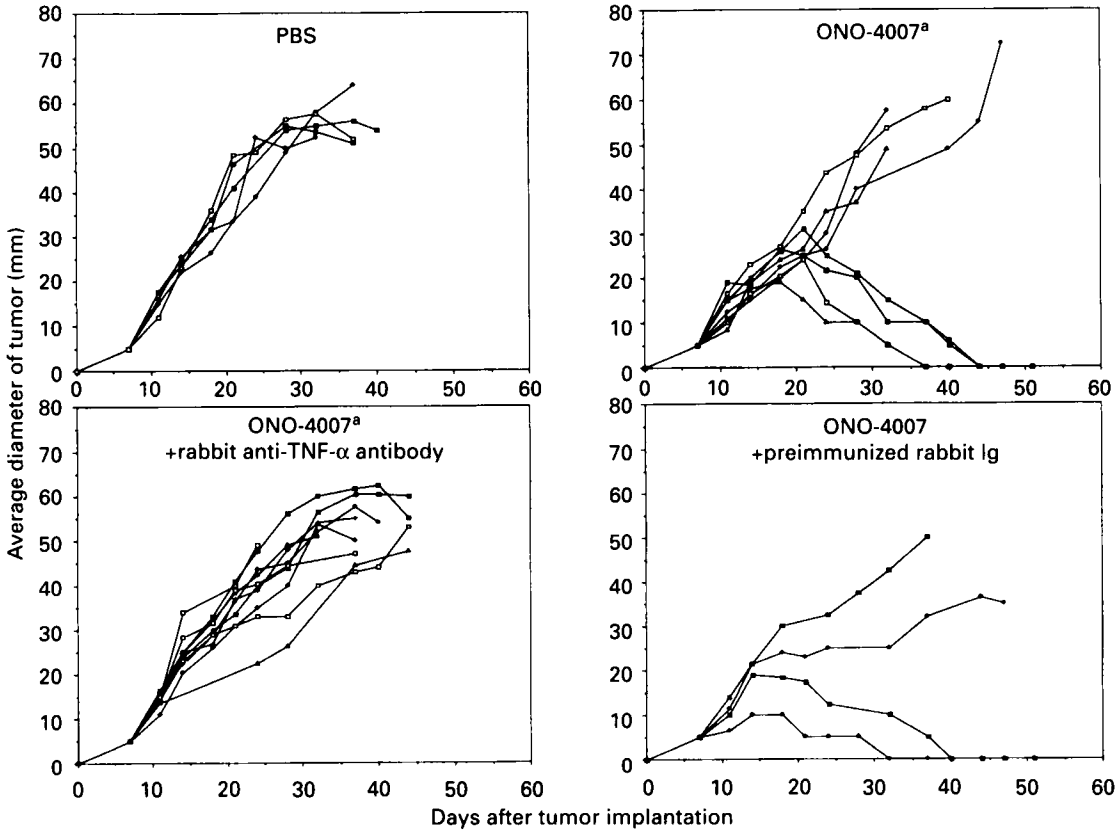

Figure 3. Abrogative effects of anti-TNF-α antibody on the *in vivo* effects of ONO-4007. KDH-8 cells (1×10^5) were implanted s.c. in WKAH rats on day 0. ONO-4007 (3.0 mg/kg) or PBS was administered i.v. four times on days 7, 14, 21 and 28. Antibody was administered i.v. four times 30 min prior to administration of ONO-4007 each time. [a]Statistically significant survival ratio ($p < 0.01$).

Table 4. Effects of anti-TNF-α antibody on the production of TNF-α and IL-1β in the tumor tissues

Treated with	Number	TNF-α	IL-1β	IFN-γ
ONO-4007	*n*=4	101.1±23.9 ng/ml	26.0±9.7 ng/ml	not detected
ONO-4007+preimmunized rabbit Ig antibody	*n*=3	69.9±25.5 ng/ml [b] [a]	20.7±2.1 ng/ml	not detected
ONO-4007+rabbit anti-TNF-α antibody	*n*=3	10.0±4.0 ng/ml	15.0±4.9 ng/ml	not detected
PBS	*n*=3	not detected	not detected	not detected

KDH-8 cells (1×10^5) were implanted s.c. in WKAH rats on day 0. ONO-4007 (3.0 mg/kg) or PBS was administered i.v. four times on days 7, 14, 21 and 28. Antibody was administered i.v. four times 30 min prior to administration of ONO-4007 each time. Rats were killed 90 min after the administration on day 28. The tumor tissues were resected and homogenized in RPMI 1640 medium supplemented with 10% FBS, 1 ml per 100 mg portion. After centrifugation at 20 000 *g* for 60 min, they were passed through 0.45 μm pore size filters and assayed by ELISA.
[a]Statistically significant ($p<0.01$).
[b]Statistically significant ($p<0.05$).

Table 5. Transplantation resistance against KDH-8 tumor cells in KDH-8-cured rats

Experiment	No. of cells challenged	Died/challenged	
		Normal	Cured
1	1×10^5	7/7	0/7
2	5×10^5	8/8	0/2
	1×10^6	ND[a]	0/2
	5×10^6	ND	0/3
	1×10^7	ND	0/2
	Total	15/15	0/16

KDH-8 cells were rechallenged when primary tumors were completely cured (on day 63). Duration between the cure and the rechallenge varied among rats. The cured rats used in this experiment were obtained from two independent experiments.
[a]Not done.

Table 6. Transplantation resistance even against TNF-α resistance cKDH-8/11 in KDH-8-cured rats

Tumor cells rechallenged	Died/used (MST±SD)[a]	
	Normal	Cured
cKDH-8/11	4/4[b] (56.3±6.8)	0/4[b] (–)
KEG-1	6/6 (64.7±5.6)	5/5 (84.4±8.6)

cKDH-8/11 and KEG-1 cells (1×10^5) were rechallenged when primary KDH-8 tumors were completely cured (on day 63–65). Duration between the cure and the rechallenge varied among rats. The cured rats used in this experiment were obtained from two independent experiments.
[a]Mean survival time of dead rats±SD.
[b]Statistically significant ($p<0.01$).

KDH-8-specific immune response in the Winn assay

As shown in Table 7 (Experiments 1–3), spleen cells of the cured rats inhibited the proliferation of only KDH-8 cells but not of KMT-17 and KEG-1 cells. Furthermore, as shown in Table 7 (Experiment 4), the growth of cKDH-8/11 cells was also inhibited. These results strongly indicated that the KDH-8-specific immune response in which TNF-α was not concerned was induced in the cured rats.

Effector cells involved in the KDH-8-specific immune response in the Winn assay

According to the above results that the specific immune response against both TNF-α sensitive KDH-8 and TNF-α resistant cKDH-8/11 cells was induced, we speculated the lymphocytes were involved as the

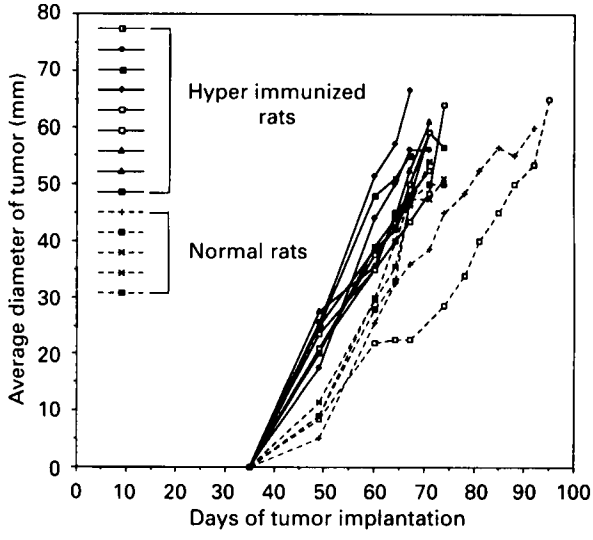

Figure 4. Growth curves of KDH-8 cells in rats hyper-immunized with MMC-KDH-8 cells. KDH-8 cells (1×10^7) were incubated with MMC (50 μg/ml) in 1 ml RPMI 1640 medium supplemented with 10% FBS at 37°C for 1 h. After washing with PBS, cells (1×10^6) were implanted s.c. in WKAH rats three times on days 0, 7 and 14 for immunization. On day 21, viable KDH-8 (1×10^5) cells were implanted s.c.

Table 7. Tumor cell neutralizing activity of spleen cells obtained from KDH-8-cured rats by the administration of ONO-4007 detected in the Winn assay

Experiment	Effector	E/T	Weight of tumor [g (% inhibition)]					
			KDH-8		KMT-17		KEG-1	
1	none		1.70±0.08[a]		4.24±1.59		2.71±0.66	
	spleen cells	100:1	0.42±0.20[a]	(75.24)	3.39±0.48	(20.04)	2.41±0.62	(10.89)
	from KDH-8-cured rats	10:1	1.61±0.10	(5.38)	3.32±0.39	(21.84)	2.57±0.40	(5.23)
	spleen cells	100:1	1.64±0.08	(3.22)	3.88±0.60	(8.63)	2.34±0.62	(13.45)
	from normal rats	10:1	1.68±0.06	(1.01)	3.43±0.33	(19.09)	2.39±0.35	(11.93)
2	none		1.61±0.09[b]		5.03±1.75		ND[e]	
	spleen cells	100:1	0.38±0.28[b]	(76.68)	4.59±1.17	(8.74)	ND	
	from KDH-8-cured rats	10:1	1.32±0.70	(17.92)	5.64±1.87	(−12.28)	ND	
	spleen cells	100:1	1.53±0.37	(5.24)	5.85±0.93	(−16.35)	ND	
	from normal rats	10:1	1.26±0.48	(21.98)	7.04±2.32	(−39.98)	ND	
3	none		0.80±0.25[c]		ND		ND	
	spleen cells	100:1	0.04±0.09[c]	(95.00)	ND		ND	
	from KDH-8-cured rats	50:1	0.31±0.19[c]	(61.88)	ND		ND	
		10:1	0.77±0.10	(4.38)	ND		ND	
	spleen cells	100:1	0.74±0.31	(10.23)	ND		ND	
	from normal rats	50:1	0.88±0.24	(−6.64)	ND		ND	
		10:1	0.98±0.06	(−18.57)	ND		ND	
4 (against cKDH-8/11)	none		1.24±0.24		−		−	
	spleen cells from KDH-8-cured rats	100:1	0[d]	(100.0)	−		−	
	spleen cells from normal rats	100:1	0.56±0.15[d]	(54.53)	−		−	

Effector cells were obtained from spleens extracted from normal rats or completely cured rats on day 63. Effector cells (1×10^7 and 1×10^8/ml) were mixed with tumor cells (1×10^6/ml, each) at an equal volume and then the mixture (0.2 ml) was implanted s.c. into syngeneic WKAH rats irradiated with 300 rad 1 day before implantation. At 14 days after tumor implantation, rats were killed and tumors were extracted. The percent inhibition was evaluated by the formula given in the text.

[a,b,c,d]Statistically significant ($p<0.01$).

[e]Not done.

effector cells in it and they might recognize a common antigen on the surface of the two cells. Then, in order to identify the effector cells, we carried out the Winn assay using a subpopulation of spleen cells and KDH-8 cells. As shown in Table 8, the CD4-rich population of spleen cells obtained from the cured rats inhibited the proliferation of KDH-8 cells as well as whole spleen cells did.

Table 8. $CD4^+$ spleen cells inhibited the growth of KDH-8 cells in the Winn assay

Effector cells (subpopulation of spleen cells)	Tumor take/used
Whole	0/5
CD4-rich population	0/5
CD8-rich population	5/5
Macrophage-rich population	4/5
None	5/5

Effector cells were obtained from spleens extracted from completely cured three rats on day 63 and mixed. Cell depletion was carried out with antibody and complement (see Materials and methods). The cell mixture of KDH-8 cells (1×10^5) and effector cells (5×10^6) was implanted s.c. into WKAH rats irradiated with 300 rad 1 day before implantation. Tumor take was evaluated 14 days after tumor implantation.

Discussion

The present study shows that ONO-4007 had therapeutic effects in half of the rats implanted with TNF-α-sensitive KDH-8 cells and that *in vivo* effects of ONO-

4007 were completely abrogated by anti-TNF-α antibody. These results are consistent with our previous report that ONO-4007 induced the production of TNF-α in tumor tissues, leading to the rejection of KDH-8 tumors.[19] They also suggest that TNF-α is an essential effector which mediates the *in vivo* therapeutic effects of ONO-4007. Furthermore, we observed that the transplantation resistance was induced in the cured rats specific to KDH-8 cells and that the transplantation resistance was mediated by $CD4^+$ cells. These findings suggest that an anti-tumor immune response following the production of TNF-α in the tumor tissues was also involved in the rejection of implanted KDH-8 cells. It remains to be elucidated how ONO-4007 induced the T-cell-mediated immune response specific to low antigenic KDH-8 cells which produced transforming growth factor (TGF)-β.[33,34] We previously reported that TGF-β derived from KDH-8 cells suppressed macrophage function and consequently suppressed immune response in tumor-bearing rats. As Mokyr *et al.* have reported that TNF-α restored the cytotoxic T lymphocytes induction inhibited by tumor-derived TGF-β, i.e. TNF-α may play an important role in the induction of the T cell-mediated immune response inhibited by TGF-β.[35] Alternatively, TNF-α may activate the antigen-presenting cells and consequently lead T cells to recognize tumor-associated antigens. In fact, TNF-α has been reported to play an important role in the development of dendritic cells that are specialized antigen-presenting cells and in the expression of B7-1 in Langerhans cells that can also act as antigen-presenting cells.[36] Furthermore, inflammatory cytokines including TNF-α have been reported to activate the adhesion molecules on the vascular endothelial cells, which consequently promotes the infiltration of various types of cells.[37–39]

Immunosuppression in the tumor-bearing state was observed frequently in the $CD4^+$ T cell-mediated immune response as well as in macrophage functions possibly related with those stated above. We previously reported that IL-2 production and TNF-α production were reduced, and that inhibition of TGF-β production restored IL-2 production and TNF-α production in KDH-8-bearing rats.[19,34,40] In this experiment we observed that $CD4^+$ T cells specific to KDH-8 cells were activated by administration of ONO-4007 in the cured rats and that $CD4^+$ T cells were responsible for the anti-tumor immune response. Our finding is consistent with the previous report that ONO-4007 restored TNF-α-production and IL-2 production, and consequently impaired delayed-type hypersensitivity in tumor-bearing mice.[11] Although we could not determine whether $CD4^+$ T cells had cytotoxicity against KDH-8 cells, the result of the Winn assay suggests that $CD4^+$ T cells have cytotoxic activity as previously reported.[41–43]

Finally we should next investigate the immune response initiated by ONO-4007 from the viewpoint of IL-12, because lipid A and its analogs are strong inducers of IL-12 from macrophages. However, some controversies remain that combination of IFN-γ is required.[44,45]

In conclusion, the present study demonstrated that ONO-4007 had therapeutic effects on rats implanted with KDH-8 cells through the production of TNF-α in tumor tissues with activation of $CD4^+$ T cells specific to KDH-8 cells.

Conclusion

ONO-4007, a new synthetic lipid A derivative with low toxic activities, had significant therapeutic effects on rat hepatocellular carcinoma KDH-8 through the production of TNF-α. This is strongly confirmed by the fact that rabbit anti-TNF-α antibody completely abrogated the therapeutic effects. Furthermore, in the cured rats, anti-tumor specific immunity was induced, which we presume was by the immune response cascade initiated by TNF-α production. The prime effector involved in the specific immune response was $CD4^+$ cells.

Acknowledgments

We thank Dr Y Kuramitsu, Dr Y Ohiro and Dr M Obara for their kind help and discussions, and Ms M Yanome for preparing the manuscript.

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(Received 4 November 1997; accepted 27 November 1997)