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Immunostimulatory action of L-4-oxalysine counteracts immunosuppression induced by α-fetoprotein

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Abstract

We have previously reported that L-4-oxalysine, a natural product in China, exhibits marked antitumor and immunoregulatory activities. The present study was set up to investigate the effect of L-4-oxalysine on the immunological activity of α-fetoprotein. It was observed in in vitro experiments that concanavalin A reactivity, one-way mixed lymphocyte reaction and interleukin-6 activity of spleen cells from hepatoma-22-bearing mice were significantly inhibited by various concentrations of α-fetoprotein. However, L-4-oxalysine functionally antagonized the α-fetoprotein-induced suppression of the mitogen- and one-way mixed lymphocyte culture-induced proliferation of spleen lymphocytes and interleukin-6 production by these cells in mice bearing the hepatoma-22 tumor. The results indicate that L-4-oxalysine has immunostimulatory activity and this effect of the agent counteracts the immunosuppression induced by α-fetoprotein, although L-4-oxalysine does not directly antagonize α-fetoprotein.

Keywords: L-4-Oxalysine; α-Fetoprotein; Mitogen reactivity; Lymphocyte reaction, one-way mixed; Interleukin-6; Liver cancer

1. Introduction

L-4-oxalysine is a natural product isolated from a new species of *Streptomyces roseoviridofuscus* n.sp. in China and its chemical formula is $NH_2–CH_2–CH_2–O–CH_2–CH(NH_2)–COOH$ (Zhang et al., 1979; Bao et al., 1981). It has been demonstrated that L-4-oxalysine inhibits the proliferation of some mouse implanted tumors and pulmonary metastasis of mouse Lewis lung carcinoma (Wang and Xu, 1996c). However, the mechanisms by which L-4-oxalysine exerts its antitumor activities are still unclear. It is known that α-fetoprotein, an oncofetal antigen, is re-expressed in large amounts in adult hepatoma cells and serves as a clinically useful marker of primary liver cancer. Although the pathological distribution and diagnostic importance of α-fetoprotein have been widely recognized, precise information concerning its various biological functions is still lacking. Our previous investigation shows that human α-fetoprotein stimulates the growth of mouse hepatoma-22 cells and that such an effect seemingly results from a direct action of α-fetoprotein on hepatoma-22 cells (Wang and Xu, 1995a). Furthermore, it is also possible that hepatoma cells escape the host antitumor defense by producing α-fetoprotein molecules that have a negative effect on the immune system (Wang and Xu, 1995b). Thus α-fetoprotein may contribute to the generation and development of primary liver cancer. Our recent investigation suggests that L-4-oxalysine is effective against hepatoma cell growth (Wang and Xu, 1996c). Preliminary clinical studies indicate that treatment with L-4-oxalysine improves symptoms of patients with primary liver cancer (Wang and Xu, 1996d). Is the anti-hepatoma activity of L-4-oxalysine associated with its effect on the biological functions of α-fetoprotein? It has been demonstrated that L-4-oxalysine has an antagonistic action on hepatoma-22 cell growth stimulated by α-fetoprotein (Wang and Xu, 1996b), and L-4-oxalysine is capable of potentiating immunological responses in tumor-bearing mice (Wang and Xu, 1996e, 1997). In the present study, we examined whether the immunostimulatory action of L-4-oxalysine counteracts the immunosuppression induced by α-fetoprotein in order to investigate the mechanisms of its therapeutic action on α-fetoprotein-associated diseases such as primary liver cancer.

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2. Materials and methods

2.1. Animals and cell lines

All animal experiments in the present study were approved by the Ethics Committee of the Chinese Academy of Sciences.

Female Institute of Cancer Research (ICR) and C57BL/6J mice were purchased from Shanghai Animal Center, Chinese Academy of Sciences (Shanghai, China) and used at ages of 6 to 8 weeks with an average body weight of 20 g. Mice were housed 4–5 per cage and given rodent chow and tap water ad libitum. MH-60 (Mouse Hybridoma-60) BSF-2 (B-cell stimulatory factor-2) cell lines were kindly supplied by Dr. Zhi-Gang Tian, Shandong Medical Academy of Sciences (Jinan, China) and cultured with RPMI 1640 media containing 20 units ml^{-1} of human recombinant interleukin-6. Mouse hepatoma-22 cell lines were maintained in RPMI 1640 media.

2.2. Drugs and reagents

L-4-oxalysine, a water-soluble white powder, was kindly provided by Department of Antibiotics of this Institute. Mitomycin C was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). HEPES was obtained from Shanghai Institute of Biochemistry, Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium, concanavalin A, lipopolysaccharide, human recombinant interleukin-6, human serum albumin, Sepharose 4B and cyanogen bromide were bought from Sigma (St. Louis, MO, USA). New-born bovine serum was produced by Second Military Medical University (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was bought from Fluka (Fluka, Switzerland) and dissolved in Hanks' balanced salts solution without phenol red. All RPMI 1640 media were supplemented with 5×10^{-5} M of 2-mercaptoethanol, 25 mM of HEPES, 2 mM of L-glutamine, 100 international units ml^{-1} of penicillin, 100 $\mu g\,ml^{-1}$ of streptomycin and heat-inactivated 10% new-born bovine serum, pH 7.4.

2.3. Tumor implantation

Mouse hepatoma-22 cells (1×10^7) were intraperitoneally implanted into the above-mentioned female ICR mice. Invariably, the transplanted tumors exhibited 100% survival. Tumor-bearing animals were housed 4–5 per cage and given rodent chow and tap water ad libitum. Animals bearing a hepatoma-22 tumor had consistently low levels of circulating α-fetoprotein in their sera. For assay of immunological responses, tumor-bearing mice were killed by cervical dislocation 7 to 10 days after implantation of tumors.

2.4. Isolation and identification of α-fetoprotein

The α-fetoprotein preparation was isolated from human umbilical cord sera by sequential precipitation with ammonium sulphate (Nishi, 1970) and purified by anti-α-fetoprotein monoclonal antibody affinity chromatography as described by Ruoslahti (1976). All purified alpha-fetoprotein samples showed only a single band of protein on polyacrylamide electrophoresis. Protein concentrations were determined according to Lowry's method.

2.5. Preparation of spleen cells

Mice were killed by cervical dislocation. Spleen was freshly removed and was gently teased apart in cold RPMI 1640 media, using a mesh net of 80-gauge stainless steel. The spleen cells were filtered through the steel sieve, washed three times in cold RPMI 1640 media, counted in 0.2% trypan blue, and adjusted to the desired cell concentration. Cell viability was usually about 98%.

2.6. Mitogen reactivity

Spleen cells were suspended at 2×10^6 cells ml^{-1} in RPMI 1640 media. Cell suspension containing 1×10^5 viable cells was incubated in 96-well flat-bottom culture plates (Costar, Cambridge, USA) with the culture media containing concanavalin A in 5% CO_2 and air at 37°C for 72 h. Preparations to be tested were added at the start of culture. Control cultures contained cells alone. Three replicates for each group were done. Total volume of each well was 100 μl. Final concentrations of spleen cells and

Table 1
Inhibition of mitogen reactivity of spleen cells by in vitro treatment with α-fetoprotein

Concentration ($\mu g\,ml^{-1}$)	Mitogen reactivity (Absorbance at 540 nm)	
	With α-fetoprotein	With human serum albumin
0	1.03 ± 0.07	1.03 ± 0.07
3.1	0.85 ± 0.02	1.01 ± 0.05
6.3	0.75 ± 0.03[a]	1.01 ± 0.07
12.5	0.62 ± 0.02[a]	1.04 ± 0.02
25.0	0.52 ± 0.04[a]	1.04 ± 0.04
50.0	0.42 ± 0.05[a]	1.03 ± 0.06
100.0	0.35 ± 0.04[a]	1.05 ± 0.05

1×10^6 cells ml^{-1} of spleen cells from hepatoma-22-bearing mice were incubated for 72 h with concanavalin A (5.0 $\mu g\,ml^{-1}$) in the absence or presence of various concentrations of α-fetoprotein or human serum albumin. Control cultures contained cells alone (absorbance at 540 nm: 0.48 ± 0.04). Mitogen reactivity was determined by MTT-microculture tetrazolium assay and results are expressed as absorbance at 540 nm. Absorbance was measured with an enzyme-linked-immunosorbent assay plate reader. Means ± S.E.M. for 6 animals.

[a] Statistically significant at $P < 0.05$ compared to 0 $\mu g\,ml^{-1}$ group.

Table 2
Stimulatory effect of L-4-oxalysine on the mitogen reactivity of spleen cells

Concentration ($\mu g\,ml^{-1}$)	Mitogen reactivity (Absorbance at 540 nm)	
	With α-fetoprotein	Without α-fetoprotein
0	0.53 ± 0.06	0.97 ± 0.09
3.1	0.57 ± 0.07	0.97 ± 0.21
6.3	0.67 ± 0.06[a]	0.96 ± 0.15
12.5	0.78 ± 0.04[a]	1.03 ± 0.03
25.0	0.76 ± 0.10[a]	1.16 ± 0.08[a]
50.0	0.86 ± 0.04[a]	1.28 ± 0.22[a]
100.0	0.95 ± 0.12[a]	1.41 ± 0.29[a]

Spleen cells were incubated for 72 h with concanavalin A ($5.0\ \mu g\,ml^{-1}$) in the absence or presence of α-fetoprotein ($25.0\ \mu g\,ml^{-1}$). Control cultures consisted of cells alone and concanavalin A. [a]Statistically significant at $P < 0.05$ compared to 0 $\mu g\,ml^{-1}$ group. The additional details see Table 1.

concanavalin A were 1×10^6 cells ml^{-1} and 5.0 $\mu g\,ml^{-1}$, respectively. MTT was added to the cultures for the last 4 h of culture. Mitogen reactivity was determined by MTT-microculture tetrazolium assay and expressed as absorbance at 540 nm.

2.7. One-way mixed lymphocyte cultures

One-way mixed lymphocyte cultures were prepared by a modification of the technique described by Murgita and Tomasi (1975b). In brief, spleen cells from C57BL/6J mice at a density of 2×10^7 cells ml^{-1} were incubated at 37°C for 30 min with 25 $\mu g\,ml^{-1}$ of mitomysin C. The cells were washed twice in cold Hanks' balanced salts solution containing 10% new-born bovine serum, incubated again for 10 min at 37°C and washed before being adjusted to a concentration of $6–8 \times 10^7$ cells ml^{-1}. These mitomysin C-treated cells served as 'stimulator' cells in one-way mixed lymphocyte cultures. The second set of spleen cells was prepared from allogeneic ICR mice bearing the hepatoma-22 tumor and adjusted to a concentration of 4×10^7 cells ml^{-1} to serve as 'responder' cells in one-way mixed lymphocyte cultures. One-way mixed lymphocyte cultures contained $3–4 \times 10^6$ stimulator cells plus 2×10^6 responder cells in 200 μl of RPMI 1640 media in 96-well flat-bottom culture plates. Control cultures contained cells from either source alone. Three replicates of each cell type or combination were made. Preparations to be tested were added at the start of culture. Plates were incubated in 5% CO_2 and air at 37°C for 72 h. MTT was added for the last 4 h of culture. Results were determined by comparing absorbance at 540 nm in responder cells incubated with stimulator cells, to absorbance at 540 nm in responder cells incubated with stimulator cells and preparations to be tested. Absorbance was measured in the MTT-microculture tetrazolium assay.

2.8. Generation of interleukin-6 and assay of its activity

As reported by Theisen-Popp et al. (1992), 100 μl of spleen cell suspension ($1–1.5 \times 10^7$ cells ml^{-1}) was added to 24-well flat-bottom culture plates (Costar, Cambridge, USA) with 50 μl of lipopolysaccharide ($125\ \mu g\,ml^{-1}$). Preparations to be tested were also added at the start of culture. The total volume of each well was 1.0 ml. Final concentrations of spleen cells and lipopolysaccharide were $1–1.5 \times 10^6$ cells ml^{-1} and 6.3 $\mu g\,ml^{-1}$, respectively. After incubation at 37°C in a 5% CO_2 incubator for 64 h, the interleukin-6-containing supernatant was collected, clarified by centrifugation ($600 \times g$, 10 min), and stored at −25°C until assayed for interleukin-6 activity.

The assay for interleukin-6 activity was performed by using an interleukin-6-dependent B-cell hybridoma, the MH-60 BSF-2 cell line (Matsuda et al., 1988). Briefly, the MH-60 BSF-2 cells were cultured in 96-well flat-bottom culture plates at a final concentration of 1×10^5 cells ml^{-1} in the presence of interleukin-6-containing supernatant. After 24 or 48 h, depending on the growth condition of MH-60 BSF-2 cells, the proliferative response of MH-60

Table 3
Inhibition of the one-way mixed lymphocyte reaction of spleen cells by in vitro treatment with α-fetoprotein

Group	Concentration ($\mu g\,ml^{-1}$)	One-way mixed lymphocyte reaction (Absorbance at 540 nm)
Stimulator cells alone	0	0.39 ± 0.13[a]
(Responder + Stimulator) cells	0	1.33 ± 0.10
α-Fetoprotein	5	1.19 ± 0.07(10.5)
α-Fetoprotein	10	0.98 ± 0.08(26.3)[a]
α-Fetoprotein	20	0.87 ± 0.07(34.6)[a]
α-Fetoprotein	40	0.72 ± 0.08(45.9)[a]
α-Fetoprotein	80	0.62 ± 0.09(53.4)[a]

Responder spleen cells (1×10^7 cells ml^{-1}) from hepatoma-22-bearing ICR mice were incubated for 72 h with stimulator spleen cells (2×10^7 cells ml^{-1}) from normal C57BL/6J mice in the absence or presence of various concentrations of α-fetoprotein. Control culture contained stimulator cells alone. One-way mixed lymphocyte reaction was determined by MTT-microculture tetrazolium assay and results are expressed as absorbance at 540 nm. The numbers in parentheses indicate the percent inhibition. Means ± S.E.M. for 6 animals.
[a]Statistically significant at $P < 0.05$ compared to (Responder + Stimulator) cell group.

Table 4
Immunostimulatory activity of L-4-oxalysine counteracts the suppression of the one-way mixed lymphocyte reaction of spleen cells induced by α-fetoprotein

Concentration ($\mu g\,ml^{-1}$)	One-way mixed lymphocyte reaction (Absorbance at 540 nm)
0	0.72 ± 0.08
6.3	0.75 ± 0.19
12.5	0.84 ± 0.06
25.0	0.89 ± 0.08[a]
50.0	0.94 ± 0.19[a]
100.0	1.11 ± 0.15[a]

Responder cells were incubated for 72 h with stimulator cells in the presence of α-fetoprotein (40 $\mu g\,ml^{-1}$). Control cultures consisted of stimulator cells alone and (Responder + Stimulator) cells.
[a]Statistically significant at $P < 0.05$ compared to 0 $\mu g\,ml^{-1}$ group. For the additional details see Table 3.

BSF-2 cells was measured by using the MTT-microculture tetrazolium assay. Interleukin-6 activity is expressed as absorbance at 540 nm.

2.9. MTT-microculture tetrazolium assay

Cellular growth in the presence or absence of experimental agents was determined by using the previously described MTT-microculture tetrazolium assay (Mosmann, 1983; Denizot and Lang, 1986). In brief, 25 μl of MTT at a final concentration of 800 $\mu g\,ml^{-1}$ was added to each well of 96-well flat-bottom culture plates for the last 4 h of culture. Four hours later, 100 μl of 10% sodium dodecyl sulphate-5% isobutanol-0.12% HCl solution was added to each well to solubilize the MTT-formazan product. Additional controls contained culture media alone. The culture plates were incubated at 37°C in a 5% CO_2 incubator for another 12 h. Absorbance at 540 nm was measured with an enzyme-linked-immunosorbent assay plate reader.

2.10. Statistical analysis

Data were analyzed by Student's *t*-test. Results are presented as means ± S.E.M. A level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Immunostimulatory action of L-4-oxalysine counteracts the suppression of α-fetoprotein on mitogen reactivity of spleen cells from hepatoma-22-bearing mice

As shown in Table 1, the proliferation of spleen lymphocytes was markedly stimulated, by 114.6%, by 5.0 $\mu g\,ml^{-1}$ of concanavalin A. α-Fetoprotein was added to the spleen cell cultures containing concanavalin A at the beginning of the experiment and spleen cells were incubated for 72 h in the presence of α-fetoprotein. It was found that α-fetoprotein at 6.3–100 $\mu g\,ml^{-1}$ clearly inhibited the mitogen reactivity of spleen lymphocytes induced by concanavalin A, the inhibition being about 27–66%. The cell viability of cultures containing α-fetoprotein did not differ from that of control cultures. In order to examine the specificity of the inhibitory action of α-fetoprotein, human serum albumin was also added at the same concentrations to the culture system at the beginning of the experiment. No suppression of concanavalin A-stimulated spleen cell mitogenesis was observed. As shown in Table 2, L-4-oxalysine at 25–100 $\mu g\,ml^{-1}$ stimulated concanavalin A-induced proliferation of spleen cells when used alone. Moreover, in the presence of α-fetoprotein (25 $\mu g\,ml^{-1}$), L-4-oxalysine at concentrations ranging from 6.3 to 100 $\mu g\,ml^{-1}$ functionally antagonized the inhibitory influence of α-fetoprotein on mitogen reactivity. The immunostimulatory potency of L-4-oxalysine in the α-fetoprotein-treated cultures was the same as that in cultures treated with L-4-oxalysine alone. It is concluded that the

Table 5
Inhibition of interleukin-6 activity of spleen cells by in vitro treatment with α-fetoprotein

Group	Concentration ($\mu g\,ml^{-1}$)	Interleukin-6 activity (Absorbance at 540 nm)
Cells alone	0	0.21 ± 0.06[a]
Lipopolysaccharide	6.3	1.15 ± 0.18
Lipopolysaccharide + α-Fetoprotein	6.3 + 6.3	0.95 ± 0.07(17.4)
Lipopolysaccharide + α-Fetoprotein	6.3 + 12.5	0.90 ± 0.06(21.7)[a]
Lipopolysaccharide + α-Fetoprotein	6.3 + 25.5	0.67 ± 0.10(41.7)[a]
Lipopolysaccharide + α-Fetoprotein	6.3 + 50.0	0.59 ± 0.06(48.7)[a]
Lipopolysaccharide + α-Fetoprotein	6.3 + 100.0	0.44 ± 0.06(61.7)[a]

1.5×10^6 cells ml^{-1} of spleen cells from hepatoma-22-bearing mice were incubated for 64 h with 6.3 $\mu g\,ml^{-1}$ of lipopolysaccharide in the absence or presence of various concentrations of α-fetoprotein. Control cultures contained cells alone. The assay for interleukin-6 activity was performed with an interleukin-6-dependent cell line, MH-60 BSF-2 and results are expressed as absorbance at 540 nm. The numbers in parentheses indicate the percent inhibition. Means ± S.E.M. for 6 animals.
[a]Statistically significant at $P < 0.05$ compared to lipopolysaccharide group.

stimulatory effect of L-4-oxalysine on mitogen reactivity of spleen cells counteracts the suppression induced by α-fetoprotein.

3.2. Immunostimulatory action of L-4-oxalysine counteracts the suppression of α-fetoprotein on one-way mixed lymphocyte reaction of spleen cells from hepatoma-22-bearing mice

Mixed spleen lymphocytes from stimulator cells and responder cells were incubated for 72 h with various concentrations of α-fetoprotein and (or) L-4-oxalysine. From Table 3, the proliferation of responder cells from hepatoma-22-bearing mice stimulated by mitomycin C-treated stimulator cells was markedly inhibited by in vitro treatment with α-fetoprotein at concentrations ranging from 10 to 80 $\mu g\,ml^{-1}$, with about 26–53% suppression. The α-fetoprotein preparation did not affect cell viability as measured by the ability of cells to exclude trypan blue. However, as shown in Table 4, L-4-oxalysine at 25–100 $\mu g\,ml^{-1}$ increased the one-way mixed lymphocyte reaction suppressed by α-fetoprotein (40 $\mu g\,ml^{-1}$) toward normal range values. It is inferred that the stimulation by L-4-oxalysine of the one-way mixed lymphocyte reaction of spleen cells can also counteract the inhibitory effect of α-fetoprotein.

3.3. Immunostimulatory action of L-4-oxalysine counteracts the suppression of α-fetoprotein on interleukin-6 activity of spleen cells from hepatoma-22-bearing mice

The interleukin-6 activity of spleen cells was significantly enhanced by in vitro treatment with lipopolysaccharide at 6.3 $\mu g\,ml^{-1}$. Spleen cells treated with 12.5–100 $\mu g\,ml^{-1}$ of α-fetoprotein had a lower interleukin-6 activity than α-fetoprotein-untreated ones, indicating that α-fetoprotein is capable of inhibiting the interleukin-6 activity of spleen cells (Table 5). No nonspecific cytotoxic influence of α-fetoprotein was observed by the trypan blue exclusion method. In the presence of α-fetoprotein (50 $\mu g\,ml^{-1}$), the α-fetoprotein-depressed interleukin-6 activity of spleen cells was enhanced by L-4-oxalysine at concentrations ranging from 6.3 to 100 $\mu g\,ml^{-1}$, indicating that L-4-oxalysine functionally antagonizes the inhibitory effect of α-fetoprotein on the interleukin-6 activity and that the stimulatory effect of L-4-oxalysine on the interleukin-6 activity of spleen cells counteracts the α-fetoprotein-induced suppression (Table 6).

Table 6
Immunostimulatory activity of L-4-oxalysine counteracts the suppression of interleukin-6 activity of spleen cells induced by α-fetoprotein

Concentration ($\mu g\,ml^{-1}$)	Interleukin-6 activity (Absorbance at 540 nm)
0	0.55 ± 0.12
6.3	0.74 ± 0.06[a]
12.5	0.78 ± 0.11[a]
25.0	0.86 ± 0.03[a]
50.0	0.94 ± 0.02[a]
100.0	1.03 ± 0.14[a]

Spleen cells were incubated for 64 h with lipopolysaccharide (6.3 $\mu g\,ml^{-1}$) in the presence of α-fetoprotein (50.0 $\mu g\,ml^{-1}$). Control cultures consisted of cells alone and lipopolysaccharide.
[a]Statistically significant at $P < 0.05$ compared to 0 $\mu g\,ml^{-1}$ group. For the additional details see Table 5.

4. Discussion

The ability of α-fetoprotein of human origin to suppress the immune responses of spleen cells from mice bearing hepatoma-22 tumors was observed in the present study. It has been shown that this embryonic substance is a potent immunosuppressor. In vitro, it inhibits lymphocyte proliferation induced by various mitogens such as phytohaemagglutinin, concanavalin A and lipopolysaccharide. The allogeneic lymphocyte stimulation in one-way mixed lymphocyte cultures, and primary and second antibody responses are also suppressed by in vitro treatment with α-fetoprotein (Alpert et al., 1978; Lester et al., 1978; Murgita and Tomasi, 1975a,b; Murgita et al., 1978). α-Fetoprotein administered in vivo also suppresses antibody formation (Orga et al., 1974). However, the exact mechanisms by which α-fetoprotein exerts its immunosuppressive activities are largely unclear. In another study, induction of suppressor T cells by α-fetoprotein was suggested to underlie its immunoregulatory mechanism (Wang and Xu, 1996a,b). The result was in agreement with those of Murgita et al. (1977) and Alpert et al. (1978). In the present work, lower interleukin-6 activity was found in α-fetoprotein-treated spleen cells, suggesting that suppression by α-fetoprotein of interleukin-6 activity may also contribute to its immunoregulatory influence.

Studies indicate that α-fetoprotein directly stimulates the growth of hepatoma-22 cells and exerts a negative effect on the host immune system. Some data suggest that α-fetoprotein receptors may exist on the surface of some cells such as immune cells and hepatoma cells. α-Fetoprotein appears to bind directly to its receptors on the cell membranes, causing a variety of changes in the biological functions of cells by affecting common signal pathways such as cyclic nucleotide systems (i.e., cAMP and cGMP) (Dattwyler et al., 1975; Mizejewski et al., 1975). It is unlikely that α-fetoprotein acts intracellularly, in view of its easy removal by washing and the lack of a subsequent effect (Lester et al., 1978). There is no evidence that α-fetoprotein merely inhibits mitogen reactivity by direct interference with mitogen or by competitive binding of the added tritiated thymidine by α-fetoprotein (Murgita et al., 1978), thereby excluding the possibility that the α-fetoprotein-induced inhibition of mitogen responses could

be due to a physical interaction between α-fetoprotein and mitogen interfering with the capacity of the mitogen to stimulate lymphocytes or competition with mitogen for cell-surface receptors. However, the exact mechanisms of the growth-stimulating and immunosuppressive action of α-fetoprotein are very complex and have not been adequately investigated to date. Therefore, further studies are necessary.

Abnormally high serum α-fetoprotein levels and its immunosuppressive action have been reported in patients with primary liver cancer (Purves et al., 1973; Murgita and Tomasi, 1975b). It is considered that these high α-fetoprotein concentrations, similar to those used to obtain in vitro immunosuppression in the present study, may be an important factor causing the nonspecific immunosuppression in patients with primary liver cancer. Furthermore, as Murgita and Tomasi (1975b) have stressed before, it is also possible that local immunosuppression in the absence of high serum levels of α-fetoprotein may also be caused by the production of suppressive concentrations of α-fetoprotein within and around the microenvironment of the disease process (i.e., the proliferating tumor cells). Therefore, it is suggested that the present in vitro results with microgram amounts of α-fetoprotein may be applicable in vivo with nanogram serum levels of α-fetoprotein in patients with primary liver cancer.

The immunostimulatory effect of L-4-oxalysine has been investigated in vitro and in vivo. In the present investigation, it was found that L-4-oxalysine had a marked stimulating effect on in vitro proliferation of spleen lymphocytes from hepatoma-22-bearing mice induced by concanavalin A. In another paper, it was observed that L-4-oxalysine alone increased the one-way mixed lymphocyte reaction and interleukin-6 activity of spleen cells from mice bearing hepatoma-22 tumors in vitro (Wang and Xu, 1997). In normal mice, L-4-oxalysine (50–100 mg kg^{-1}) caused a pronounced increase in the number of spleen plaque-forming cells and in the serum complement C3, hemolysin and Ig G content. The mitogen reactivity of spleen lymphocytes and sheep red blood cell-induced delayed-type hypersensitivity are also promoted by in vivo treatment with L-4-oxalysine (Li et al., 1987). In brain tumor-22-bearing mice, in vivo administered L-4-oxalysine (200 mg kg^{-1}) exhibited potent antitumor immunity in Winn's assay (Wang and Xu, 1996e). Preliminary clinical investigation indicates that oral treatment with L-4-oxalysine (10–20 mg kg^{-1}) can ameliorate immunodeficiency in patients suffering from primary liver cancer (Li et al., 1987). It is concluded that although it is difficult to compare the doses of L-4-oxalysine in vivo with the concentrations used in vitro, the in vivo results are very similar to the in vitro ones. The immunostimulatory action of L-4-oxalysine was further demonstrated in the present in vitro experiments with α-fetoprotein. The results indicate that concanavalin A-induced mitogen reactivity, one-way mixed lymphocyte reaction and lipopolysaccharide-induced interleukin-6 activity of spleen cells from hepatoma-22-bearing mice suppressed by α-fetoprotein were significantly enhanced by L-4-oxalysine. Our previous study also showed that L-4-oxalysine inhibited the suppressor T cell activity induced by α-fetoprotein in hepatoma-22-bearing mice (Wang and Xu, 1996a,b). These results suggest that L-4-oxalysine has potent immunostimulatory activity, and although L-4-oxalysine has no direct antagonistic effect with regard to α-fetoprotein, its immunostimulatory action counteracts the immunosuppression induced by α-fetoprotein.

As reported previously, L-4-oxalysine inhibits the growth of hepatoma-22 cells (Wang and Xu, 1996c). L-4-oxalysine can also antagonize hepatoma-22 cell growth stimulated by α-fetoprotein (Wang and Xu, 1996b). It is known that the chemical structure of L-4-oxalysine is similar to L-lysine, suggesting that there may be an interaction between L-4-oxalysine and L-lysine. Our previous studies also indicate that the suppression of tumor growth and fibrinogen synthesis caused by L-4-oxalysine could be significantly antagonized by co-administration of L-lysine (Wang and Xu, 1996e). Therefore, although the exact mechanism(s) of the antitumor action of L-4-oxalysine cannot be defined from the available data, an interesting speculation which merits consideration is that the synthesis of some L-lysine-enriched proteins in hepatoma cells may be inhibited by competition between L-4-oxalysine and L-lysine. A clear therapeutic effect of L-4-oxalysine has been demonstrated in patients with primary liver cancer without any apparent side effects (Wang and Xu, 1996d). L-4-oxalysine has a protective influence against liver injuries induced by carbon tetrachloride, and the activity of glutathione *S*-transferase in mouse liver tissue decreased by carbon tetrachloride is increased after co-administration of L-4-oxalysine (Zeng et al., 1982). It has also been found that L-4-oxalysine lowers serum glutamic–pyruvate transaminase levels in patients with chronic persistent hepatitis (Wang and Xu, 1996d). The pharmacokinetics of L-4-oxalysine suggest that the agent is higher in liver than in other tissues (Wang and Xu, 1996d). Taken together, these results imply that L-4-oxalysine can be developed to become a new drug for the treatment of tumors, especially primary liver cancer.

Interleukin-6 has been identified as a growth and differentiation factor for immune cells and plays an important role in the immune response (Kishimoto, 1989). Thus, the L-4-oxalysine-stimulated enhancement of the interleukin-6 activity of spleen cells from mice bearing the hepatoma-22 tumor suppressed by α-fetoprotein may be associated with its antitumor activity. However, the function of interleukin-6 is not restricted to immune cells but includes a wide variety of biological activities in various tissues and cells. Some studies have demonstrated that interleukin-6 stimulates the proliferation of certain malignant cells such as plasmacytoma/myeloma cells; however the growth of other tumor cells such as myeloid leukemia cells and breast carcinoma cells is suppressed by interleukin-6

(Kishimoto, 1989). Further investigations will be undertaken to determine the action of interleukin-6 on hepatoma cell proliferation and the possible influence of α-fetoprotein or L-4-oxalysine on interleukin-6 activity.

In conclusion, the findings presented in this paper show that L-4-oxalysine exhibits immunostimulatory activity and that this effect of the agent counteracts the immunosuppression induced by α-fetoprotein. Immunotherapy with various biological response modifiers has become a new area of tumor therapy. This therapy mobilizes the natural defense factors against tumors, and, compared with conventional chemotherapy, it has fewer side effects. Thus it is considered that the immunostimulatory effect of L-4-oxalysine, which is one of the most important functions of the agent, may enhance the immunological defense of the tumor-bearing host, and thus L-4-oxalysine may be a potential biological response modifier in immunotherapy for cancers which are associated with the expression and release of α-fetoprotein.

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