ACTIVATION OF NATURAL CYTOTOXIC ACTIVITY AND CONCOMITANT REDUCTION OF TRIGLYCERIDE CONTENT OF MURINE SPLEEN, TREATED WITH AN ANTITUMOR ANTIBIOTIC, ASCOFURANONE

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Ascofuranone (AF) elevated natural cytotoxic activity of spleen when it was administered intraperitoneally to male mice. The elevation was observed both in low and high responder mice. AF-activated splenocytes lysed NK-resistant tumor cells, FM3A, P388 and sarcoma 180 cells as well as NK-sensitive YAC-1 cells. However, AF suppressed other lymphatic functions such as mitogenic responses and interleukin 2 production. Because AF did not activate splenic NK activity *in vitro*, the activation is assumed to be caused by a host-mediated process. One of the possibilities is modulation of the lipid metabolism of splenocytes. Thus, we examined splenic lipid contents and revealed that AF decreased splenic triglycerides without affecting other lipids. In contrast, the antibiotic significantly increased triglyceride in muscle.

Ascofuranone (AF) was isolated from a phytopathogenic fungus *Ascochyta viciae* as a hypolipidemic substance, having prenyl-phenolic structure[1]. It reduces serum lipid level of experimental animals after oral administration[2]. It also modulates lipid metabolism of leukemia L5178Y cells *in vitro* and alters membrane properties[3].

Recently, we reported its antitumor activity against experimental tumors, including L1210, sarcoma 180 and Ehrlich carcinoma. A characteristic of the antitumor activity is that AF is effective with pretreatment as well as with post-treatment, indicating that the antitumor activity is host-mediated[4]. As AF modulates lipid metabolism, the host-mediated antitumor activity may be caused by modulating lipid metabolism.

In this report, we studied the effects of AF on the host immune system and revealed an activation of splenic natural cytotoxicity and concomitant reduction of the triglyceride content by AF.

Materials and Methods

Mice

C3H/He, BALB/c, BDF1, *dd*Y and C57BL/6, 6~10 weeks old male mice were purchased from Shizuoka Experimental Farm (Hamamatsu). Commercial pellet diet (CE-2, Clea, Japan, Ltd., Tokyo) and tap water were fed *ad libitum*.

Chemicals

Purified ascofuranone (AF) was supplied by Chugai Pharmaceutical Co., Ltd. (Tokyo). For *in vitro* studies, AF was dissolved in methanol at 10 mg/ml and diluted to appropriate concentration with medium. For *in vivo* administration, AF was suspended in 0.5% Tween 80 dissolved in phosphate-buffered saline (PBS, pH 7.4) with the aid of a Teflon homogenizer. RPMI1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% fetal bovine serum (FBS, Flow Laboratories, North

Ryde, N.S.W. Australia), 5×10^{-5} M 2-mercaptoethanol, 50 μg/ml kanamycin and 5 mM HEPES was used for cell culture. EAGLE's MEM (Nissui Seiyaku Co., Tokyo) without serum was used to wash the cells.

Assay of Cytotoxic Activity

A suspension of spleen cells treated with ammonium chloride to remove erythrocytes was prepared as described previously[4]. Target cells in late log phase were collected and incubated for 45 minutes in 50 μl of 1 mCi/ml [^{51}Cr]sodium chromate saline solution (New England Nuclear, Boston, MA). After three washes with MEM, 1×10^4 target cells were incubated with effector cells in 200 μl volume at 37°C in a flat-bottomed microplate well (Nunc, Roskilde, Denmark). After 4-hour incubation, the radioactivity in 100 μl of supernatant was determined. % Lysis was calculated by the following equation:

% Lysis=(Experimental release − Spontaneous release)/(Maximum release − Spontaneous release)

Spontaneous and maximum releases were determined by culturing the target cells in medium and in 0.5% sodium dodecyl sulfate solution, respectively.

Production of Interleukin 2 (IL 2) and its Assessment

Five million splenocytes in 1 ml volume in a flat-bottomed 16-mm diameter plastic well (Nunc, Roskilde, Denmark) were cultured in the presence of 5 μg/ml concanavalin A (con A, Sigma Chem., St. Louis, MO). After 24-hour culture at 37°C, the supernatants were collected and centrifuged to remove contaminated cells. They were stored at −20°C until used. IL 2 activity in the supernatant was measured by a proliferative response of thymocytes in the presence of suboptimal concentration of phytohemaggultinin (PHA). One million C3H/He thymocytes prepared in the same manner as splenocytes except the removal of erythrocytes, in 200 μl volume in a flat-bottomed microplate well were cultured in the presence of 10% supernatant and 1 μg/ml PHA (Wellcome, Bechenham, UK). After a 44-hour culture, each well was pulsed with 0.5 μCi [^{3}H]thymidine (New England Nuclear, Boston, MA). Cultures were harvested 4-hour later by an automatic cell harvester.

Extraction and Estimation of Lipid Content

About 100 mg slices of muscle or liver was carefully dissected and homogenized by a glass homogenizer in 20 volumes of chloroform - methanol (2: 1). After 20 hours, the precipitate was removed by filtration and the extract was stored at −20°C until used. Blood was removed from the hearts of ether-anesthetized mice, using a heparinized syringe and plasma was obtained by centrifugation. Lipids in extracts and plasma were determined by the following assay methods: Triglycerides by VAN HANDEL method[5]; phospholipids by ZILVERSMIT method[6]; free fatty acid by ITAYA-UI method[7] and total cholesterol by ZURKOWSKI's method[8].

Results

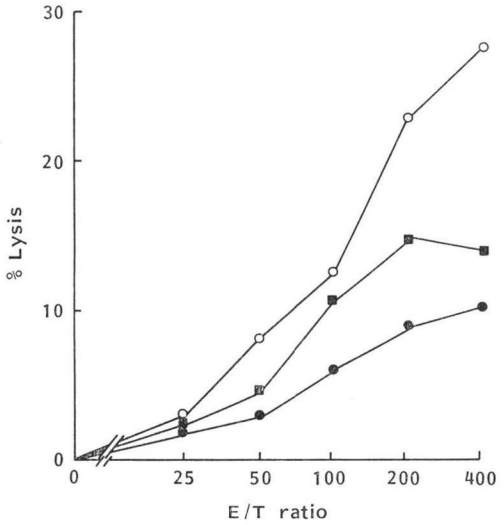
Activation of Natural Cytotoxic Activity of Spleen

AF augmented splenic natural cytotoxic activity when administered intraperitoneally 7, 5 and 3 days before the assay (Fig. 1). Activation was observed with all the effector-to-target (E/T) ratios tested. Cytotoxic capacity of splenocytes reached to plateau level at E/T=200 and AF elevated the plateau level. This suggests that the activity of each effector cell was amplified. AF stimulated cytotoxic activity in a dose-dependent manner and a relatively high dose was required for significant activation. LD_{50} of AF was about 800 mg/kg.

As shown in Table 1, normal mice of C3H/He and C57BL/6 strains had higher endogenous cytotoxic activity than *dd*Y, BALB/c and BDF1. AF activated splenic cytotoxic activity of both types of mice. Maximum activation was observed with BDF1 mice, which reached more than 4.5-fold over the control. In addition, splenocytes from AF-treated mice were able to lyse natural killer (NK)-resistant tumor cells (P388, FM3A and sarcoma 180) as well as NK-sensitive YAC-1 (Table 2). It is

Fig. 1. Activation of splenic cytotoxic activity by AF.

Splenocytes from three mice treated ip with 200 (■) and 400 (○) mg/kg of AF, 7, 5 and 3 days before the assay were incubated with ^{51}Cr-labeled YAC-1 cells at the indicated E/T ratios. Control mice (●) received only the vehicle.



also noted that they were able to lyse the cells of sarcoma (sarcoma 180) and carcinoma (FM3A) as well as lymphoma (YAC-1).

In Vitro Effect of AF on Splenic Cytotoxic Activity

Some substances that activate NK cells *in vivo* also activate them *in vitro*[9]. However, preincubation of splenocytes with AF had no influence on cytotoxic activity. Rather, high concentration of AF suppressed it (Table 3). In addition, cytotoxicity of splenocytes was not elevated when cytotoxic assay was performed in the presence of AF (data not shown). Therefore, activation of splenic cytotoxicity by AF is assumed to be host-mediated.

Table 1. Activation of splenic cytotoxic activity of AF-administered mice.

Mouse strain	% Lysis	
	Control	AF-treated
BALB/c	7.1	32.0[b](451)[a]
BDF1	10.1	47.1[b](466)
C3H/He	24.3	65.8[b](271)
C57BL/6	19.1	40.0[b](209)
*dd*Y	7.5	34.1[b](455)

One million splenocytes from three mice treated ip with 200 mg/kg of AF, 7, 5 and 3 days before the assay were cultured with 1×10^4 ^{51}Cr-labeled YAC-1 cells in triplicate cultures. Four hours later, the radioactivity released in the supernatant was determined. Control mice received only the vehicle. Standard deviation of each assay was within ten percent of average value.

[a] Figures in parentheses represent % to control value.

[b] Statistically significant by Student's test at $P<0.001$.

Table 2. Target spectrum of AF-activated splenocytes.

Target cells	% Lysis	
	Control	AF-treated
FM3A	7.8	16.6[b](212)[a]
Sarcoma 180	8.9	24.3[b](274)
P388	5.1	22.0[b](433)
YAC-1	33.6	64.1[b](191)

One million splenocytes from three C3H/He mice treated ip with 200 mg/kg of AF, 7, 5 and 3 days prior the assay were cultured with 1×10^4 ^{51}Cr-labeled target cells in triplicate cultures. Four hours later, the radioactivity in the supernatant was determined. Control mice received only the vehicle. Standard deviation of each assay was within ten percent of control value.

[a] Figures in parentheses represent % to control value.

[b] Statistically significant by Student's test at $P<0.05$.

Major cell population that contribute to splenic natural cytotoxicity is NK cells and typical intrinsic activators of NK cells are interleukin 2 (IL 2) and interferon (IFN)[10]. As T cells produce both factors, activation of T cells would result in the activation of NK cells. However, splenocytes from AF-treated mice showed poorer responses to PHA and con A, which are polyclonal activators of T cells. In fact, splenocytes from AF-treated mice produced less IL 2 than the control in response to con A (Table 4). The similar results were obtained when IL 2 activity was determined by IL 2 dependent lymphocyte line. Therefore, it is likely that AF activated splenic cytotoxicity through

Table 3. Inhibition of splenic cytotoxic activity by the preincubation with AF.

AF (μg/ml)	% Lysis
0	36.9
0.6	46.5
1.3	32.5
2.5	34.0
5	5.0[a]
10	0[a]

Ten million C3H/He splenocytes per ml were cultured in the presence of indicated concentration of AF for 24 hours. Cells were washed three times with MEM and 1×10^6 viable cells were cultured with 1×10^4 ^{51}Cr-labeled YAC-1 cells in triplicate assays. Four hours later, radioactivity in the supernatant was determined. Standard deviation of each assay was within ten percent of control value.

[a] Statistically significant by Student's test at $P<0.001$.

other mechanism than the activation of T cells.

Table 4. AF-treated mice is prevented to release IL 2 in response to con A.

Group	[^{3}H]Thymidine incorporation[a] (cpm)
Experiment 1	
Control	42,742±2,125
AF-treated	33,824±250[c]
Experiment 2	
Control	40,179±7,562
AF-treated	16,032±2,281[c]
Medium[b]	10,524±1,926

Five million C3H/He splenocytes per ml from three mic etreated with 200 mg/kg of AF, 7, 5 and 3 days before the assay were cultured with 5 μg/ml con A for 24 hours. The IL 2 activity in the supernatant was assessed as described in Materials and Methods. Control mice received only the vehicle.

[a] Mean±SD of triplicate assays.

[b] Incorporation of [^{3}H]thymidine into thymocytes in the absence of exogenous IL 2.

[c] Statistically significant by Student's test at $P<0.001$.

Effects of AF on Splenic Lipid Content

As AF affects lipid metabolism both *in vivo* and *in vitro*, this property could be related to activation of splenic natural cytotoxic activity. We, therefore, estimated the lipid contents of spleens from AF-treated mice to ascertain whether AF also affects the lipid metabolism of the lymphoid organ. The result is shown in Table 5. AF significantly reduced splenic triglycerides (TG) in a dose dependent manner whereas no influence on other major lipid content, free fatty acids (FFA), phospholipids (PL) and cholesterol, was observed. To ascertain whether the influence on TG content is specific for spleen, TG content of serum and liver was also estimated and it was found to be affected only slightly. However, muscle TG increased more than two-fold (Table 6), indicating that AF modulates lipid metabolism systemically.

Discussion

As lipid is one of the major components of plasma membrane, it greatly affects the property of the membrane[11,12]. Immunological interactions take place on the plasma membrane. Therefore, cellular lipid metabolism is assumed to have profound influence on an immune network. In addition, alteration of phospholipid metabolism is reported in various immune reactions including activation of NK cells[13], macrophages[14] and polymorphonuclear leukocytes (PMN)[15], which are possible mediators of natural cytotoxic activity of spleen. Therefore, it is interesting to study how an immune network is affected by AF which modulates lipid metabolism both *in vivo* and *in vitro*.

We found that AF reduced splenic TG content concomitantly with the activation of natural cytotoxicity. Further study is needed to explore the relationship between activation of natural cytotoxic activity and the reduction of TG content in spleen of AF-treated mice. However, we have obtained a preliminary result that an immunopotentiator, OK-432, which activates splenic NK activity[16], also greatly affects splenic lipid metabolism. Our results obtained in the present report strongly suggest that lipid metabolism and an immune system intimately correlate with each other.

Possible populations mediating splenic natural cytotoxicity include NK cells, macrophages and PMNs. In 4-hour cytotoxic assay, however, cytotoxic activity may be mainly contributed by NK cells

Table 5. Effect of AF on splenic lipid contents.

AF (mg/kg)	Lipid content[a] (mg/g)			
	TG	FFA	PL	Cholesterol
0	6.27±0.14	12.2±1.9	0.68±0.02	1.00±0.03
100	4.84±0.21[b]	11.8±3.5	0.62±0.05	0.95±0.01
	(77)	(97)	(91)	(95)
200	5.15±0.20[b]	9.4±7.0	0.60±0.01[b]	0.93±0.07
	(82)	(77)	(89)	(93)
400	4.32±0.44[b]	12.7±2.4	0.63±0.01[b]	0.96±0.03
	(69)	(104)	(91)	(96)

Lipid was extracted from the spleens of three mice treated with indicated dose of AF, 7, 5 and 3 days before the sacrifice. Each lipid content was determined in the triplicate assays.

[a] Mean±SD. Figures in parentheses represent % to control value.

[b] Statistically significant by Student's test at $P<0.01$.

Table 6. Effect of AF on plasma, liver and muscle TG content.

AF (mg/kg)	Triglyceride content[a]		
	Plasma (mg/dl)	Liver (mg/g)	Muscle (mg/g)
0	34.2±4.4	1.38±0.20	7.8±0.6
100	27.9±1.3	1.55±0.15	13.9±0.9[b]
	(82)	(112)	(178)
200	47.2±2.2	1.69±0.12	21.4±1.4[b]
	(138)	(122)	(274)
400	29.9±2.3	1.30±0.10	12.8±0.3[b]
	(87)	(94)	(164)

Lipid content of tissues and plasma from three mice treated with indicated dose of AF, 7, 5 and 3 days before the sacrifice, was determined in triplicate assays.

[a] Mean±SD. Figures in parentheses represent % to control value.

[b] Statistically significant by Student's test at $P<0.001$.

because the expression of complete cytotoxicity of macrophages[17] or PMNs[18] requires more than 10 hours of incubation. Furthermore, AF-activated splenocyte preferentially lyse NK-sensitive target, YAC-1 cells. However, they also attacked the tumor cells which belong to the NK-resistant group. In our preliminary results, the cytotoxic effects belonged to the non-adherent population, when spleen cells were separated into adherent and non-adherent populations by passing through a Sephadex G-10 column. However, the method could not completely exclude macrophages and especially neutrophiles when cell populations were morphologically determined by Giemsa staining. Therefore, our present results cannot completely rule out the possibility of participation of macrophages and PMNs. In fact, we found that PMNs increased in the splenocytes of AF-treated mice (MAGAE, J.; K. NAGAI & G. TAMURA: unpublished results). Expansion of target spectrum by AF might be due to the PMNs. Final characterization of effector cells will be accomplished by application of specific antibody.

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