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Effect of Flavone Acetic Acid (NSC 347 512) on Splenic Cytotoxic Effector Cells and their Role in Tumour Necrosis*

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Abstract—*Flavone acetic acid (FAA), an antitumour agent currently undergoing clinical trial, has immune-modulatory effects on various cytotoxic cells in mice. Natural killer (NK) cell activity in the spleen was augmented* 4 h *after FAA treatment, and when spleen cells were cultured with interleukin-2 to induce the production of lymphokine-activated-killer (LAK) cells, higher levels of LAK cell activity were generated by spleen cells from FAA-treated animals than by spleen cells from untreated, control mice. The response to FAA by spleen cells from mice bearing the Colon* 38 *tumour was compared to that of non-tumour bearers. Activity against NK-sensitive YAC-*1 *tumour targets was augmented to a similar degree, and no activity against NK-resistant P*815 *targets was detected. FAA was shown to induce haemorrhagic necrosis in the P*815 *tumour grown as a subcutaneous solid tumour. Furthermore, haemorrhagic necrosis was induced by FAA on Colon* 38 *tumours growing in mice which had been depleted of NK activity by treatment with anti-asialo GM-*1 *antibody. Thus, although NK activity could be involved in the long-term host response to the tumour, it does not appear to be a major determinant of FAA-induced haemorrhagic tumour necrosis.*

INTRODUCTION

FLAVONE ACETIC ACID (FAA), a flavonoid synthesized by Atassi *et al.* [1] and subsequently found to have notable activity against murine solid tumours [2], has recently undergone phase I clinical trials [3] and is now being assessed against a spectrum of human tumours. FAA has been shown experimentally to have potent *in vivo* activity against a variety of murine tumour lines [4, 5] as well as against human tumour xenografts in nude mice [5]. While FAA does have demonstrable *in vivo* cytostatic activity against human and murine tumour lines [5–8], this activity is generally quite weak. The kinetics of FAA-induced killing of a murine Lewis lung-derived tumour line *in vivo* and *in vitro* were found to be quite different [5]. The difference, together with other observations [5], raises the question of whether FAA mediated its anti-tumor effects *in vivo* indirectly, perhaps through the activation of host cytotoxic mechanisms.

A number of features place FAA in the category of biological response modifiers. Its action resembles that of tumour necrosis factor (TNFα) in inducing rapid haemorrhagic necrosis of solid tumours *in vivo* [9]. Natural killer (NK) cell activity in spleen [10], liver and peritoneum of mice [11] is augmented after FAA treatment. Finally, FAA enhances the tumorical activity of murine peritoneal macrophages in culture [12]. In this communication, we have examined the effects of FAA on cytotoxic effector cells present in the spleen and assess the role of NK cells in FAA-induced tumour necrosis.

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MATERIALS AND METHODS

Materials

FAA was obtained from the National Cancer Institute, U.S.A., through the courtesy of Dr K.D. Paull and dissolved in 5% sodium bicarbonate (33 mg/ml). Antibody to asialo-GM-1 glycosphingolipid (anti-GM-1) was purchased from Wako Pure Chemicals Industries Ltd., Osaka, Japan, dissolved in phosphate buffered saline (0.25 mg/ml) and injected i.v. (intravenously). Recombinant human interleukin-2 (IL-2) from Immunex, Seattle, U.S.A., was obtained through the courtesy of Professor J.D. Watson, Department of Immunobiology, University of Auckland Medical School. Sodium polyinosinate:polycytidylate (poly I:C) (Sigma

Chemical Co., U.S.A.) was dissolved in physiological saline (1 mg/ml) and injected i.p. (intraperitoneally).

Culture medium (αMEM; Gibco, Grand Island, NY) was supplemented with foetal bovine serum (10%, Gibco NZ Ltd), 2-mercaptoethanol (50 μM) and antibiotics (penicillin 100 units/ml, streptomycin sulphate 100 μg/ml). Multiwell culture plates were obtained from Linbro, Flow Lab, U.S.A.

Mice

$C_{57}BL/10J$ (B10) or $(C_{57}BL/2J \times DBA/2J)F_1$ (BDF_1) mice (stocks originating from Jackson Laboratory, Bar Harbor, U.S.A.) between 8 and 12 weeks of age were bred in laboratory animal facilities under constant temperature and humidity with sterile bedding and food.

Tumour lines

Colon 38 tumour stocks were obtained in 1981 from Mason Research Institute, Worcester, U.S.A. and after *in vivo* passage were stored under standard conditions at −196°C. Tumours were passaged s.c. (subcutaneously) in $C_{57}BL/6J$ mice and grown for experiments in BDF_1 or B10 mice. Tumours were grown to a diameter of approx. 10 mm in carrier mice, removed surgically and cut into 1 mm^2 fragments for implantation in recipient mice anaesthetized by i.p. injection of with pentobarbital (90 mg/kg, dissolved in 0.2 ml physiological saline). P815 mastocytoma was obtained from Dr J. Marbrook, Department of Immunobiology, University of Auckland Medical School, and was maintained in culture as well as *in vivo* as an ascites in BDF_1 mice. Solid tumours were obtained by s.c. implantation of 10^6 P815 cells from the ascites fluid of P815 carrier mice. YAC-1 lymphoma cells were maintained in culture for use as NK-sensitive targets.

Histology

Groups of mice with s.c. Colon 38 or P815 tumours (4–8 mm diameter) were killed by nitrogen narcosis. Tumours were removed immediately, fixed in 10% formalin and embedded in paraffin wax. Sections were stained with haematoxylin and eosin by standard methods.

Generation of lymphokine-activated killer (LAK) cells

LAK cells were generated according to the method of Rosenstein *et al.* [13]. Mice were killed by cervical dislocation, their spleens removed, teased out into culture medium, aspirated to give a single cell suspension and transferred into a tube and held for 5 min to allow any clumps to settle out. Viable white cells, distinguished by their ability to exclude eosin red, were counted in a haemocytometer. Spleen cells were cultured in 24 well flat-bottomed plates (10^7 cells per 2 ml culture) with 1000 units/ml human recombinant IL-2. Cells were harvested after 4 days in culture and tested for cytotoxic activity.

Cytotoxicity assay

Cytotoxic effector cell activity was measured using a 4 h ^{51}Cr-release assay [14]. Lysis over a range of effector to target (E:T) ratio was measured for all experiments. Cells to be tested were incubated with ^{51}Cr-labelled tumour target cells (5×10^3 per well) in V-bottomed 96-microwell plates in 0.2 ml culture medium at 37°C under a humidified atmosphere containing 5% CO_2. ^{51}Cr-labelled tumour targets were prepared by incubating cells for 45 min at 37°C with 200 μCi sodium ^{51}Cr-chromate in saline, and then washing three times. The radioactivity released after 4 h was measured by removing 0.1 ml of the supernatant and counting in a gamma counter (LKB Wallac 1270 Rackgamma 11, Wallac, Finland). The percentage lysis was calculated as [(experimental release—spontaneous release)/(maximum release)] × 100. Groups containing spleen effector cells were carried out in quadruplicate and the mean and standard error determined.

RESULTS

Enhancement of splenic NK and LAK cell activity by FAA treatment in mice

Mice were injected with FAA (330 mg/kg), spleens were removed after 4 h and the cells assayed for cytotoxicity against NK-sensitive YAC-1 targets and the NK resistant P815 targets [15] using a standard *in vitro* ^{51}Cr-release assay for cytotoxic lymphoid effector cells [13, 14, 16]. The results (Fig. 1) extend earlier findings that splenic NK activity is augmented as early as 4 h after FAA treatment [10]. Lysis of YAC-1 targets by spleen cells from FAA-treated mice was significantly higher than those from untreated mice. However, activity against P815 targets was not observed by fresh spleen cells from either FAA-treated or control animals (Fig. 1).

Cytotoxicity against P815 targets was induced when spleen cells were cultured in the presence of IL-2 for 4 days and then assayed for lytic activity (Fig. 2). As NK cells are potentiated in culture in the presence of IL-2 [16], YAC-1 targets would detect both the activity of NK cells as well as the activity of LAK cells generated during the 4 days of culture with IL-2. Lysis of P815 targets, however, would measure only LAK cell activity, as the P815 is resistant to NK cells [15]. Figure 2 shows that greater activity is detected against YAC-1 than P815 cells. It also indicates that spleen cells from mice treated 4 h previously with FAA (330 mg/kg) generate greater activity against both YAC-1 and

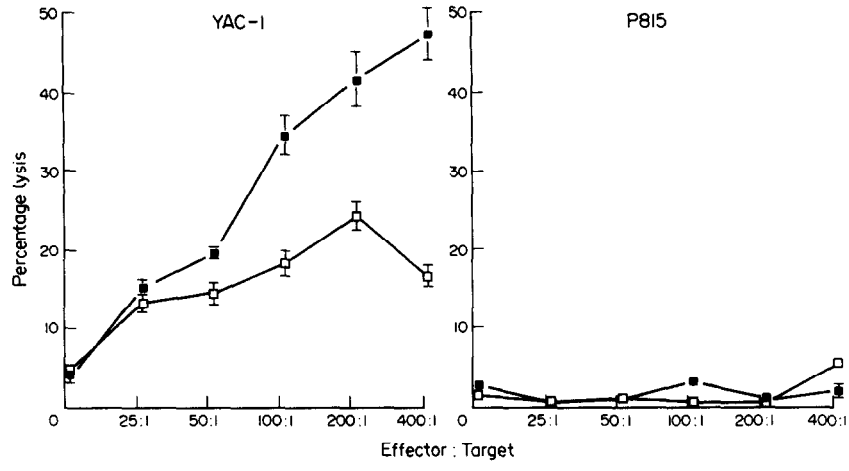

Fig. 1. Augmentation of NK cell activity. Spleen cells from BDF_1 mice treated with FAA (330 mg/kg *i.p.*) 4 h *previously (closed circles) or untreated control mice (open circles) were assayed for cytotoxic activity at varying E:T cell ratios.*

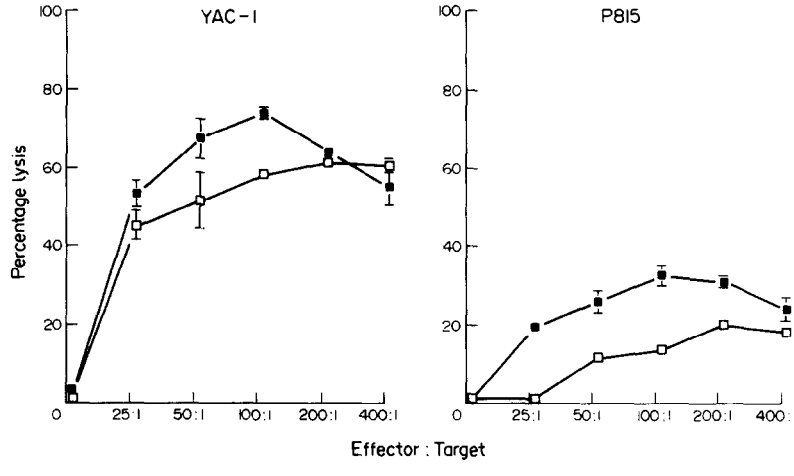

Fig. 2. Augmentation of LAK cell generation. Spleen cells from BDF_1 mice treated with FAA (330 mg/kg *i.p.*) 4 h *previously (closed circles) or untreated control mice (open circles) were cultured 4 days with IL-2. Cells harvested from the cultures were assayed for cytotoxic activity.*

P815 targets after 4 days of culture with IL-2, as compared with cultures of spleen cells from untreated mice. At optimal E:T cell ratios, enhanced cytotoxic activity was consistently observed in four separate experiments by IL-2 cultured spleen cells from FAA-pretreated mice over untreated mice.

Are NK cells involved in FAA-induced tumour necrosis?

The presence of NK activity in normal spleen, and the elevation of this activity after FAA treatment (Fig. 1; [10]) raises the possibility that FAA-induced tumour necrosis may be mediated by NK cells. Experiments were carried out to test this possibility.

We examined first whether the effector cell population in the spleens of tumour-bearing mice had in any way been altered or impaired by the presence of the tumour. Mice bearing s.c. implanted palpable Colon 38 tumours were treated with FAA (330 mg/kg). Spleens were removed from these and control mice after 6 h and the cells assayed for cytolytic activity against YAC-1 and P815 targets. The results (Table 1) show no difference in the response of the tumour bearers compared to that of normal mice. Activity against YAC-1 targets was enhanced to the same extent after FAA treatment in both control and tumour-bearing mice, and no activity against P815 was detectable in either group, with or without FAA treatment. Thus, the presence of a s.c. Colon 38 tumour did not appear to have altered the status of cytotoxic effector cells in the spleen.

If NK cells played a primary role in FAA-induced tumour necrosis, it would follow that only NK-sensitive tumours would be susceptible to FAA treatment, and NK-resistant tumours such as the P815 would not be affected. The P815 tumour was implanted s.c. in mice. When the tumours were

Table 1. Augmentation of splenic NK activity in control and tumour-bearing mice

	Percentage lysis*	
	YAC-1	P815
Normal spleen, untreated†	10.0 ± 0.4	0 ± 0
Normal spleen, FAA, 330 mg/kg, 6 h	26.6 ± 1.6	0 ± 0
Colon 38-bearer spleen, untreated‡	14.3 ± 1.6	0 ± 0
Colon 38-bearer spleen, FAA, 330 mg/kg, 6 h	23.7 ± 1.0	0 ± 0

*E:T cell ratio 200:1.
†From BDF_1 mice.
‡BDF_1 Colon 38 bearing mice.

Table 2. Lack of effect of FAA on NK sensitivity of target cells

	Percentage lysis	
Target	YAC-1	P815
YAC* (cultured)	17.1 ± 1.8	19.7 ± 5.5
P815 (cultured)	0.7 ± 0.1	0.5 ± 0.5
P815 (ascites)	0.5 ± 0.5	0 ± 0

Spleen cells from FAA-treated (330 mg/kg i.p. 4 h) B10 mice assayed at 200:1 E:T. Cultured target cells were exposed to FAA 80 μg/ml for 4 h.
*Target cells grown as an ascitic tumour were exposed to FAA (330 mg/kg) by i.p. injection 4 h before removal.

palpable, mice were treated with FAA (330 mg/kg) and the tumours assessed histologically for necrosis after 24 h as previously described [4, 5]. FAA was found to induce necrosis of tumour cells over each histological section. The appearance of the necrotic cells was similar to that observed with Colon 38 tumours.

It was possible that prior exposure to FAA rendered the P815 tumour cells sensitive to NK cells. To rule out this possibility, P815 cells were treated with FAA and then tested as targets for NK cells. As can be seen in Table 2, exposure to FAA either *in vivo* or *in vitro* did not induce P815 cells to become NK-sensitive. Furthermore, pre-treatment of YAC-1 cells with FAA did not alter their sensitivity to NK cell lysis (Table 2). The results show that NK-resistant tumours are sensitive to FAA treatment and that there is no correlation between NK sensitivity and FAA sensitivity.

We next tested whether FAA induced necrosis of tumours in mice which had been depleted of NK activity. NK cells express on their surface the glycosphingolipid asialo-GM-1, and i.v. administration of the antibody anti-GM-1 has been shown to reduce splenic NK activity [17]. Groups of seven mice containing palpable s.c. Colon 38 tumours were injected i.v. with anti-GM-1 (50 μg/mouse) to deplete NK cells, and then treated with FAA (330 mg/kg) 3 days later. The spleen and tumours were removed 4 h after FAA treatment. Spleen cells were assayed for NK activity and histological sections of the tumours were assessed for necrosis.

Table 3. Antitumour activity of FAA in NK-depleted mice

	NK activity in spleens*	Tumour necrosis†
Untreated mice		
No FAA	25.9 ± 2.7	–
FAA, 330 mg/kg, 4 h	43.1 ± 2.3	+
Anti-GM-1 treated (50 μg/mouse)		
No FAA	7.1 ± 1.1	–
FAA, 330 mg/kg, 4 h	10.1 ± 2.5	+

*Percentage lysis of YAC-1 targets at 400:1 E:T ratio by pooled spleen cells from each group (seven mice/group).
†Sections of individual Colon 38 tumours were assessed histologically for haemorrhagic necrosis; results are shown in Fig. 3.

Pre-treatment with anti-GM-1 significantly diminished NK activity in the spleen (Table 3). However, the histological appearance of sections of tumours from anti-GM-1-treated mice (Fig. 3b) was indistinguishable from that of control mice (Fig. 3a). The histology of Colon 38 tumours removed from FAA-treated mice was similar whether or not the mice had been previously administered anti-GM-1 (Fig. 3c, 3d). FAA-induced tumour necrosis in animals treated 4 h previously was not as advanced as that after 24 h [4], but was clearly present. In another experiment, tumours from mice either untreated or treated 3 days previously with anti-GM-1 (50 μg/mouse) were examined histologically 24 h after treatment with FAA (330 mg/kg). Tumours from both groups of FAA-treated mice, but not those from control and anti-GM-1 treated groups, were found to undergo extensive haemorrhagic necrosis.

Mice bearing palpable s.c. Colon 38 tumours were also treated with poly I:C (100 μg/mouse), a treatment which induces splenic natural killer activity in spleen [10]. Tumours were removed 24 h later for histological examination. No evidence of induced haemorrhagic necrosis was observed, indicating that elevation of NK activity alone does not induce tumour necrosis.

DISCUSSION

Although the mechanism of action of FAA has yet to be defined, it does not appear to act like clinical cytotoxic antitumour agents. Several observations have suggested that FAA might mediate its antitumour action indirectly through the activation of host-mediated immune cytotoxic mechanisms [5]. In support of this notion, FAA has been demonstrated to possess immune modulatory properties.

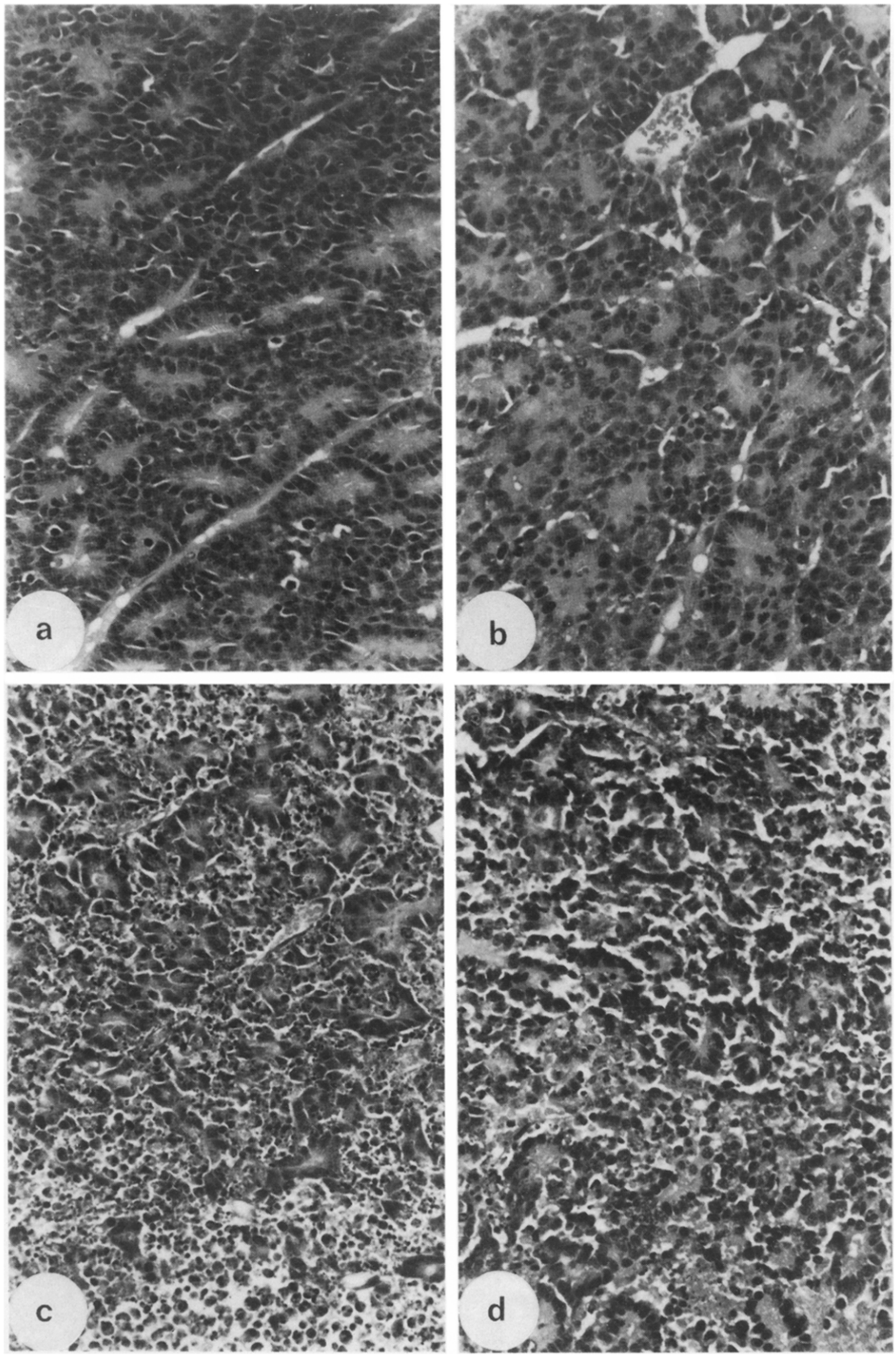

Fig. 3. Histological appearance of sections of s.c. Colon 38 tumours from untreated mice (a), mice treated 3 days previously with anti-GM-1 (b), mice treated i.p. 4 h previously with 330 mg/kg FAA (c) and mice treated with both anti-GM-1 and FAA (d). Tumours from control mice and mice treated with anti-GM-1 (a, b) were similar and showed numerous tumour acini and blood vessels. In the tumours of mice in the FAA-treated groups (c, d) there was a loss of definition of acinar structure extending over the whole section, with some rounding up of individual tumour cells and the apperaance of spaces between adjacent cells. Necrotic cells with fragmented or pyknotic nuclei were widespread through the section, and areas of spontaneous tumour necrosis could be distinguished from FAA-induced necrosis. Haemorrhagic infiltration of tumour tissue was also evident.

In vivo administration of FAA induces high levels of interferon production [18]. Systemic NK activity is enhanced after treatment with FAA *in vivo* [10, 11], and the tumoricidal activity of peritoneal macrophages in culture is augmented by the addition of FAA [12]. The results in Fig. 2 show that another class of effector cells, the LAK cells, are also affected by FAA treatment. Spleen cells taken from FAA-treated mice generated higher levels of LAK cell activity upon stimulation with IL-2 than did spleen cells from untreated control animals.

The main aim of the present experiments was to establish whether or not splenic cytotoxic effector cells were involved in FAA-induced tumour necrosis. It was possible that the presence of tumour in the host might have evoked the endogenous production of lymphokines and LAK cells. However, LAK cell activity was not detectable in spleens of tumour-bearing (Colon 38) mice from FAA-treated normal or tumour-bearing mice (Table 1). Although FAA was shown to enhance LAK cell generation in response to IL-2 (Fig. 2), NK cells appeared more likely candidates to test as effectors of FAA-induced tumour necrosis since LAK cell activity appeared only after exposure to exogenous IL-2 and could not be detected in fresh splenocytes. NK cells have been postulated as having a primary role in tumour surveillance and control of metastases [19]. Moreover, the time course for enhancement of NK activity [10] was very similar to the time course of induction of tumour necrosis by FAA [4]. However, the following points argue against the significant involvement of NK cells in the induction of haemorrhagic tumour necrosis by FAA.

(i) FAA induces haemorrhagic necrosis of the s.c. implanted P815 tumour. NK cells have activity against a restricted number of tumours, and the P815 mastocytoma line used is not sensitive to NK lysis. If FAA-induced tumour necrosis is mediated by NK cells, then only NK-sensitive tumours should be affected by FAA treatment.

(ii) NK activity can be fully induced without concomittant induction of haemorrhagic necrosis of s.c. Colon 38 tumours. Poly I:C (100 μg/mouse) and FAA (330 mg/kg) are equally effective in inducing NK activity [12], but only FAA induces tumour necrosis. Furthermore, FAA at a lower dose (100 mg/kg) efficiently induces NK activity [12] but has no effect on the histological appearance of Colon 38 tumours (results not shown).

There seems to be no correlation between splenic NK activity and FAA-induced induction of necrosis. FAA-induced haemorrhagic necrosis of the Colon 38 tumour occurs equally in groups of normal mice and groups of mice whose NK activity had been reduced more than 4-fold by treatment with anti-GM-1. Tumours contain NK activity [18] and it is possible that anti-GM-1 pre-treatment has different effect on tumour NK activity. The question of tumour associated cytotoxic effector cells will be addressed in a separate communication but it appears that anti-GM-1 pre-treatment depresses tumour cytolytic activity to one third [20].

Wiltrout and co-workers [19] have shown that FAA, like poly I:C [20], induces the production of interferons α and β *in vivo*, and that modulation of NK activity occurs via the induction of interferons. We have compared the abilities of FAA and poly I:C to augment NK activity in cultured splenic cells. Whereas poly I:C augments NK activity both *in vivo* and *in vitro*, FAA is effective only *in vivo* and does not enhance NK activity *in vitro* (Ching and Baguley, manuscript in preparation). These differences indicate that FAA and poly I:C have different mechanisms. If modulation of NK activity occurs via interferon induction [21], the lack of *in vitro* activity of FAA indicates that it must induce interferon production indirectly, in contrast to poly I:C. One possible explanation is that FAA induces macrophages or other cells to release lymphokines which in turn induce interferon production by lymphocytes. The tumoricidal activity of peritoneal macrophages can be enhanced *in vitro* by FAA [12] and it is possible that the production of lymphokines is also enhanced. We are currently investigating the mechanism of macrophage activation by FAA, as well as the involvement of macrophages in the antitumour activity of FAA.

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