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Enhancement of *In Vitro* Cytotoxicity of Mouse Peritoneal Exudate Cells by Flavone Acetic Acid (NSC 347512)*

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Abstract—*Flavone acetic acid (FAA), an antitumour agent currently undergoing clinical trials, was found to augment the tumoricidal activity of peritoneal exudate (PE) cells* in vitro. *Lysis of tumour targets was measured using a standard* 18 h ^{51}Cr *release assay for activated macrophages. Lytic activity increased with increasing concentrations of FAA up to* 100 μg/ml *before reaching a plateau. At* 80 μg/ml *FAA, 3-fold fewer PE cells were necessary to obtain the same level of activity as in control cultures without FAA. The lytic activity was mediated by Thy-1 negative and glass-adherent cells in the PE population, and was inhibited by dexamethasone. The activity of PE cells against several different tumour targets (P815 mastocytoma, YAC-1 lymphoma, P388 lymphoma, and a Lewis lung carcinoma cell line) were all enhanced by FAA. The results show that FAA can enhance the lytic potential of peritoneal macrophages* in vitro *to kill a range of tumour cells.*

INTRODUCTION

FLAVONE-8-ACETIC ACID (FAA), a new antitumour agent [1, 2] presently undergoing clinical trial [3], has a broad range of activity against advanced solid tumours in mice, but only low activity towards transplantable murine leukaemias [2, 4]. Its cytotoxic action differs from that of anti-cancer agents in clinical use in that it induces rapid and extensive haemorrhagic necrosis of susceptible tumours [4, 5]. FAA has demonstrable cytostatic activity *in vitro* [6, 7] but its weak *in vitro* activity correlates poorly with the powerful *in vivo* antitumour effects [5]. The histological appearance of *in vivo* FAA-treated tumours, as well as the kinetics of onset of damage [4], resembles that of tumour necrosis factor (TNFα) [8], suggesting the involvement of a host-mediated mechanism of toxicity. Although there is evidence that FAA has the properties of an immune-modulator in that natural killer (NK) cell activity in spleen [9, 10] and other organs [10] of mice is augmented after FAA treatment, no link to its action to TNF release has been published so far. TNF is a product of activated macrophages and in this paper we present evidence that FAA enhances the tumoricidal activity of peritoneal macrophages *in vitro*.

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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. Paull. It was dissolved in 5% sodium bicarbonate and diluted to appropriate concentrations in culture medium. Dexamethasone was obtained from David Bull Laboratories, Australia. The culture medium was αMEM (Gibco, Grand Island, NY) supplemented with foetal bovine serum (10%, Gibco, N.Z. Ltd), 2-mercaptoethanol (50 μM) and antibiotics (penicillin 100 units/ml, streptomycin sulphate 100 μg/ml).

Mice

C57Bl/10J (B10) or (C57Bl/6J × DBA/2J)F_1 (BDF1) mice between 6 and 12 weeks of age were bred in laboratory animal facilities under constant temperature and humidity with sterile bedding and food.

Peritoneal exudate (PE) cells

Mice were sacrificed by cervical dislocation, and PE cells collected by washing the peritoneum with

5 ml culture medium. Viable cells were distinguished using an eosin exclusion method. Non-adherent PE cells were prepared by incubating 5×10^7 PE cells in 5 ml medium for 1 h at 37°C in a 100 mm glass Petri dish. Medium and non-adherent cells were aspirated and placed in another Petri dish for 1 h at 37°C. Cells which did not adhere after two cycles were collected and used for the experiment. Adherent PE cells were prepared by plating appropriate numbers of PE cells directly into flat-bottomed 96-microwell trays (Linbro, Flow Lab, McLean, VA) and incubating for 2 h at 37°C. The supernatant and non-adherent cells were then removed by completely inverting the microwells. Fresh medium was added and similarly removed, and the cells which remained attached to the bottom of the microwells were used for experiments. No adjustment to the cell number was made for loss of the non-adherent cells, and the cell number given is the number of PE cells initially plated.

Depletion of Thy-1 positive cells

Thy-1$^+$ cells were removed by treatment with anti-Thy-1 antibody in the presence of complement (c′). The monoclonal anti-Thy-1 antibody used was ascites fluid from mice previously inoculated with T24/31.7 hybridoma cells [11] and was obtained through the courtesy of Dr M.A. Skinner, Department of Immunobiology, University of Auckland Medical School. The source of c′ was guinea-pig serum which had been absorbed on mouse thymocytes. Cells were placed with anti-Thy-1 (1/10 final dilution) for 45 min on ice, washed, and then incubated with c′ (1/10 final dilution) for 45 min at 37°C.

Tumour target cells

Unless otherwise stated, the target cell used was P815, which is resistant to lysis by NK cells [9]. Other lines used were YAC-1 lymphoma P388 leukaemia and LLTC carcinoma [12], all maintained in culture. Cells were ^{51}Cr-labelled by incubating for 45 min at 37°C with 200 μCi sodium ^{51}Cr-chromate in saline, and washing three times.

Cytotoxicity assay

PE cells were incubated for 18 h with labelled tumour target cells in 0.2 ml culture medium in 96-well microtitre plates at 37°C under a humidified atmosphere with 5% CO_2. 5×10^3 targets were used per well and the effector to target (E:T) ratio was varied by changing the number of PE cells. The radioactivity released 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 of lysis was calculated as [(experimental release − spontaneous release)/maximum release] × 100. All groups were carried out in quadruplicate, and the mean and standard error determined.

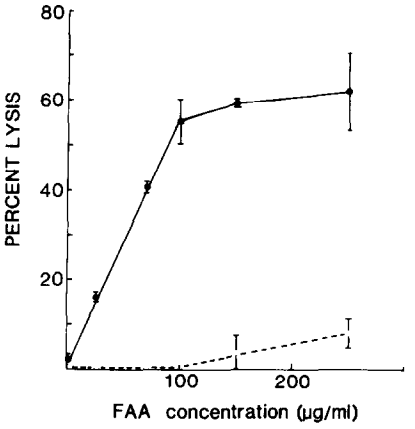

Fig. 1. Enhancement of cytotoxic activity of PE cells by FAA at different concentrations. PE cells from BDF1 mice were used at 100:1 E:T cell ratio. ^{51}Cr released from labelled P815 target was measured after 18 h. Cultures of PE cells, FAA and P815-^{51}Cr (●); no PE cells, FAA and P815-^{51}Cr only (○)

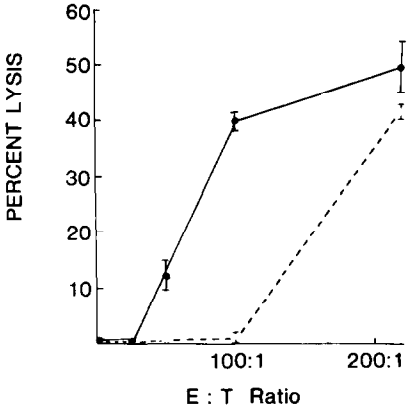

Fig. 2. FAA enhancement of PE cell cytotoxicity. BDF1 PE cells were cultured for 18 h with P815-^{51}Cr and either with (●) or without (○) 80 μg/ml FAA.

RESULTS

Effect of FAA on lysis of target cells by PE cells

PE cells were incubated for 18 h with ^{51}Cr-labelled P815 targets at an E:T cell ratio of 100:1 in the presence of varying concentrations of FAA. Lysis of P815, as measured by the amount of ^{51}Cr released, increased linearly with increasing concentrations of FAA up to 100 μg/ml, and then reached a plateau (Fig. 1). Some direct toxicity of FAA was observed at concentrations above 100 μg/ml, but this was very low compared with the activity induced in the cultures with PE cells.

Varying numbers of PE cells were incubated in the presence and absence of 80 μg/ml FAA (Fig. 2). At this dose there was no direct toxicity of FAA on P815 targets and good stimulation of PE cells was

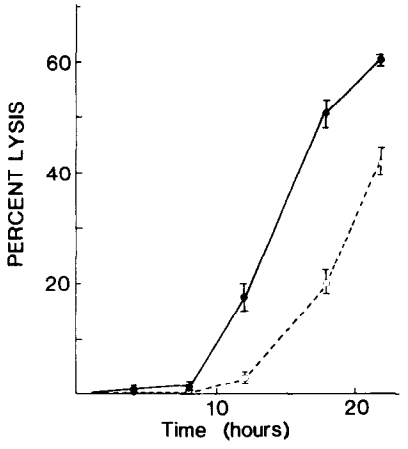

Fig. 3. Time course of lysis. B10 PE cells incubated with P815-^{51}Cr, 150:1, E:T ratio and lysis measured at various times with (●) or without (○) 80 μg/ml FAA.

obtained. PE cells at high numbers killed P815 targets on their own, but this background level of activity was significantly enhanced by addition of FAA. Without FAA, an E:T ratio of approx. 160:1 was required for 20% lysis of the targets. In the presence of FAA, the same level of lytic activity was obtained at E:T ratio of 60:1 (Fig. 2).

Time course of target cell lysis

Tumoricidal macrophages and NK cells are both able to lyse tumour cells without prior sensitization, and both cell types can be found in the PE [13]. As the P815 cell line is an NK-resistant target [9], it is unlikely that the activity demonstrated in the previous experiments was due to NK cells. Lysis by NK cells [14], as well as by other classes of lymphoid effector cells, lymphokine-activated killer cells (LAK) [15] and cytotoxic T-lymphocytes (CTL), are all detectable after 4 h in a ^{51}Cr release assay [16], whereas macrophage-mediated toxicity requires a longer incubation time [17, 18]. To distinguish between the lymphoid-mediated and macrophage-mediated cytotoxicity, the time-course of lysis of P815 targets by PE cells cultured both with and without FAA was examined.

Minimal activity was detectable in the first 8 h (Fig. 3). Significant lysis, both for the background and FAA-enhanced cultures did not occur until 12 h, and the amount of lysis was still increasing after 22 h.

Evidence for the involvement of macrophages

Since the above time-course of effect was consistent with proposal that the FAA-enhanced activity of PE cells is due to macrophages, the adherence and Thy-1 characteristics of the cells responsible for the cytotoxic activity were examined. Macrophages adhere to glass or plastic and it has been shown that over 90% of the adherent cells from the PE

Table 1. Activity in adherent cell populations

	Percentage of lysis*	
	With FAA†	Without FAA
PE‡ (unfractionated)	35.1 ± 1.1	9.0 ± 1.3
Adherent PE	29.5 ± 4.7	15.1 ± 3.7
Non-adherent PE	0 ± 0	0 ± 0

*Against P815-^{51}Cr, 18 h.
†80 μg/ml.
‡B10 PE cells, 100:1 E:T ratio.

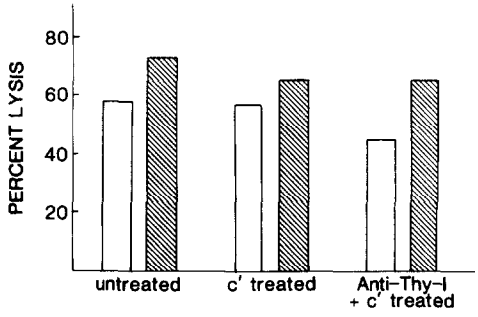

Fig. 4. Effect of anti-Thy-1 and c' treatment. PE cells from B10 mice at 150:1 E:T ratio. Lysis of P815-^{51}Cr after 18 h is shown with (hatched bars) or without (open bars) 80 μg/ml FAA.

population are macrophages [17, 18] whereas lymphoid cells are non-adherent. As can be seen in Table 1, all tumoricidal activity of PE cells was present in the adherent cell fraction and no activity was observed in the non-adherent cells.

Since all the lymphoid cytotoxic effector cells express Thy-1 on their cell surface [14–16], the effect of pretreatment of PE cells with anti-Thy-1 antibody and c' was examined. No depletion of cytotoxicity towards P815 cells was observed, in either background or FAA-enhanced activity (Fig. 4). As a positive control for the effectiveness of the anti-Thy-1 and c' treatment in removing Thy-1$^+$ cells, thymocytes were treated alongside the PE cells. The viability of untreated, c'-treated and (anti-Thy-1 plus c')-treated thymocytes was 95%, 96% and 13% respectively. The viability of untreated, c'-treated and (anti-Thy-1 plus c')-treated PE cells was 100%, 96% and 89% respectively. These results indicate that Thy-1$^+$ cells are not involved in the lysis of P815 targets by the PE cells in culture.

Dexamethasone, which is a powerful inhibitor of macrophage-mediated antitumour activity [19], inhibited both the background and FAA-enhanced cytotoxicity of the PE cells at concentrations above 10 nM (Table 2). Since dexamethasone in combination with FAA over the same concentration range was not toxic in the absence of PE cells, its action appeared to be exerted directly on the PE cell population.

Table 2. Inhibition by dexamethasone

Drug	Percentage of lysis* With PE†	Without PE
None	46.42 + 5.69	0 ± 0
FAA‡	62.25 + 1.76	0 ± 0
FAA + Dexa. 10^{-6} M	0 ± 0	0 ± 0
FAA + Dexa. 5×10^{-7} M	0 ± 0	0 ± 0
FAA + Dexa. 10^{-7} M	0 ± 0	0 ± 0
FAA + Dexa. 5×10^{-8} M	0 ± 0	0 ± 0
FAA + Dexa. 10^{-8} M	43.24 ± 6.43	0 ± 0

*P815 targets, 18 h.
†250:1 E:T ratio, B10 PE cells.
‡80 μg/ml.

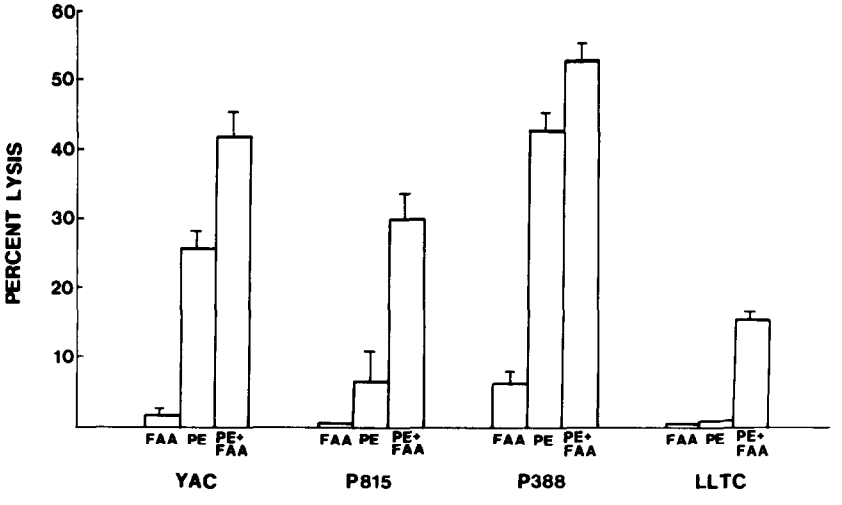

Fig. 5. Activity against a range of tumours. BDF1 PE were incubated with ^{51}Cr labelled tumour target 100:1 E:T ratio. Lysis was measured after 18 h.

Target range of antitumour activity of FAA-stimulated PE cells

FAA at 80 μg/ml enhanced the activity of PE cells against YAC lymphoma, P388 leukaemia and LLTC, a line derived from the Lewis lung carcinoma [12], as well as P815 mastocytoma, although the degree of lysis varied with the tumour cell line (Fig. 5). This broad antitumour activity is also consistent with macrophage tumoricidal activity.

DISCUSSION

When FAA was added to the cultures of PE cells taken from normal, unpretreated mice, using a standard 18 h ^{51}Cr-release assay for tumoricidal macrophage activity and an E:T ratio of 100:1, P815 target cell lysis was dramatically stimulated. Target lysis increased linearly with increasing concentrations of FAA up to 100 μg/ml, then reached a plateau level (Fig. 1). FAA alone had no direct toxicity at 100 μg/ml. Some target cell lysis was observed when PE cells were incubated in the absence of FAA with P815 cells at E:T ratios of greater than 100:1 (Fig. 2). Such activity could be due to a background of activated macrophages resident in the peritoneum, or alternatively, to effector cells which had been activated *in vitro* upon exposure to P815 cells during the 18 h incubation period. At 80 μg/ml FAA, 3-fold fewer PE cells were required to produce the same cytolytic activity as cultures without FAA (Fig. 2).

Incubation of target cells with PE cells and FAA for at least 12 h was required to produce detectable tumoricidal activity (Fig. 3), and activity was completely inhibited by dexamethasone at nanomolar concentrations (Table 2). Characterization studies showed that the effector cells were Thy-1 negative (Fig. 4) and belonged to the adherent fraction of the PE population (Table 1). Activity against a broad range of tumours was detected (Fig. 5). All these observations are consistent with the effector cells being macrophages.

Both background and FAA-stimulated cytolytic activity appear to be mediated by the same cell type. Both require a long incubation period, are

inhibitable by dexamethasone, and are mediated by Thy-1 negative cells which adhere to glass. Although the results suggest that FAA enhances the tumoricidal activity of peritoneal macrophages, it is not yet established whether the macrophages can be activated by exposure to FAA in the absence of tumour targets, or whether both are required to be present at the same time. However, this *in vitro* system will facilitate further studies on the mechanism of action of FAA.

The antitumour activity of macrophages is mediated by TNF [8], the production of which is inhibited by dexamethasone [19]. Lysis of P815 targets by PE cells in culture is completely inhibited by dexamethasone (Table 2), but attempts to demonstrate an active factor in culture supernatants have so far been unsuccessful (unpublished). However, the appearance of FAA-treated tumours *in vivo* resembles that of TNF treated tumour [4].

The *in vivo* mechanism of action of FAA on susceptible solid tumours is not understood. We have previously suggested that FAA has the properties of an immune modulator, inducing a host-mediated mechanism of cytotoxicity [4, 9]. It has been shown that NK activity is augmented after FAA treatment *in vivo* [9, 10]. The results presented here demonstrate that macrophage activity can also be enhanced by FAA. Thus, FAA appears to have a broad immune-modulatory activity, stimulating both lymphoid and macrophage cytotoxic effector cells. We are currently investigating whether or not these immune cells are involved in FAA-induced tumour regression *in vivo*.

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