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Induction of Natural Killer Cell Activity by the Antitumour Compound Flavone Acetic Acid (NSC 347 512)

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Abstract—*Flavone acetic acid (FAA), an antitumour drug at present undergoing clinical trial for cancer treatment, has been found to activate natural killer cell activity in spleen cells of mice following* in vivo *treatment at doses of* 45–330 mg/kg. *The activity of FAA was measured using a* ^{51}Cr-*release assay, employing the YAC-1 lymphoma line as a target cell population, and was found to be comparable to that of the interferon inducer polyinosinic acid–polycytidylic acid. The induction of activity was blocked by anti-asialo GM1 antibody. The time course of induction of activity by FAA was similar to that of the induction of haemorrhagic necrosis of the colon* 38 *tumour in mice, suggesting that natural killer cells or other components of the immune system may be involved in the action of FAA.*

INTRODUCTION

FLAVONE ACETIC ACID (FAA; NSC 347 512) is an antitumour compound synthesized by LIPHA (Lyons, France) and screened by Atassi *et al.* [1] and found to have high activity against subcutaneously inoculated colon 38 tumour in mice [2, 3]. FAA, together with its diethylaminoethyl ester (NSC 293 015), are currently undergoing clinical trials [4] and the mode of their antitumour action is of considerable interest. Work in this laboratory has shown that FAA and NSC 293 015 induce rapid and progressive necrosis of tumours as early as 6 h after treatment [5]. The effect is unlike that of at least the majority of clinical antitumour agents, and suggests the possibility of a host-mediated mechanism of toxicity. In the course of investigation of possible immune mechanisms of cell killing, we obtain evidence, which we now present here, that FAA stimulates natural killer (NK) activity in mice, and in that regard is as potent as the interferon inducer polyinosinic–polycytidylic acid (poly I : C).

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MATERIALS AND METHODS

Animals and therapeutic agents

All experiments were carried out using 6-week-old BDF_1 hybrid mice (C57Bl/6J × DBA/2J) bred under conditions of constant temperature and humidity using sterile bedding and food. FAA (obtained from the National Cancer Institute, U.S.A. through the courtesy of Dr. Kenneth Paull), was dissolved in 5% sodium bicarbonate and injected intraperitoneally (i.p.). Poly I : C (sodium salt; Sigma, St Louis, MO) was dissolved at 1 mg/ml and injected i.p. at 100 μg/mouse. Anti-asialo GM-1 antibody was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan, dissolved at a concentration of 0.25 mg/ml in phosphate buffered saline, and injected intravenously (0.2 ml/mouse).

Measurement of NK activity

Spleen cells from two mice of each group were pooled and assayed for NK activity using a standard 4 h ^{51}Cr-release assay [6] in Alpha modified Minimal Essential Medium (Gibco, Grand Island, NY) supplemented with foetal bovine serum (10%, Gibco NZ Ltd.), 2-mercaptoethanol (50 μM), L-glutamine (220 μg/ml) and HEPES buffer (20 mM). The YAC-1 lymphoma cell line was used as the NK sensitive targets and P815 mastocytoma cells were used as NK resistant targets. Both cell lines were maintained in culture. Quadruplicate

Table 1. Augmentation of NK activity by FAA

Treatment	% Cytotoxicity ± S.E. YAC-1 100 : 1*	30 : 1	10 : 1	P815 100 : 1	30 : 1	10 : 1
None (normal control)	14.9 ± 0.2	8.6 ± 0.6	4.8 ± 0.4	0.05 ± 0.03	0.4 ± 0.4	0.15 ± 0.08
FAA (12 h)	24.2 ± 2.0	11.9 ± 0.7	5.9 ± 0.4	2.6 ± 0.3	0.6 ± 0.6	0.7 ± 0.3
Poly I : C (24 h)	40.2 ± 0.8	18.5 ± 0.4	11.5 ± 0.5	6.1 ± 0.5	2.5 ± 0.5	1.7 ± 0.5

*Effector to target cell ratio.

Table 2. Time course of induction of NK activity

Time (h)	Inducing agent	Pre-treatment of mice	% Cytotoxicity ± S.E. 100 : 1	30 : 1	10 : 1
	None (normal control)	—	20.2 ± 0.8	8.8 ± 0.3	5.2 ± 0.8
	—	Anti-GM-1†	4.3 ± 0.9	2.4 ± 0.8	2.1 ± 0.2
1	FAA	—	21.9 ± 1.5	11.3 ± 0.6	7.4 ± 0.5
	Poly I : C	—	19.7 ± 0.7	12.6 ± 0.4	7.6 ± 1.2
2	FAA	—	21.0 ± 1.9	9.6 ± 1.5	5.6 ± 0.5
	Poly I : C	—	27.7 ± 1.3	12.8 ± 0.8	12.1 ± 1.0
4	FAA	—	32.1 ± 1.6	13.7 ± 0.7	9.9 ± 2.2
	Poly I : C	—	31.8 ± 1.0	14.2 ± 0.6	10.2 ± 0.3
24	FAA	—	31.6 ± 0.5	18.6 ± 1.5	11.2 ± 0.4
	FAA	Anti-GM-1	2.8 ± 0.3	4.7 ± 2.1	1.9 ± 0.7
	Poly I : C	—	38.8 ± 0.9	22.2 ± 1.1	12.7 ± 0.5
	Poly I : C	Anti-GM-1	4.7 ± 1.0	1.4 ± 0.5	1.2 ± 0.5
	FAA + poly I : C	—	44.8 ± 1.1	24.2 ± 0.5	17.1 ± 0.5
	None (normal control)		19.2 ± 1.5	12.3 ± 0.8	8.2 ± 1.0
4	FAA		27.5 ± 1.8	16.5 ± 0.8	8.6 ± 0.9
8	FAA		24.2 ± 0.8	14.1 ± 0.8	9.2 ± 1.0
12	FAA		26.7 ± 0.8	18.1 ± 0.9	9.9 ± 0.8
24	FAA		31.0 ± 1.8	21.0 ± 0.9	13.7 ± 1.1
	Poly I : C		34.3 ± 1.0	23.4 ± 1.3	17.1 ± 1.3

*Effector to target cell ratio.
†Mice were assayed for NK activity 4 days after intravenous injection of anti-asialo GM-1 antibody.

wells in V-bottom 96-well plates (Linbro, Flowlabs, McLean, Virginia) containing ^{51}Cr-labelled target cells (5×10^3 cells per well), and spleen cells at effector to target cell ratios of 100 : 1, 30 : 1 and 10 : 1 in a volume of 0.2 ml were incubated for 4 h at 37°C. Supernatant (0.1 ml) was then removed and the amount of radioactivity was measured using a gamma counter (LKB Wallac 1270 Rackgamma II, Wallac, Finland). The percentage of cytotoxicity was calculated as [(experimental release−spontaneous release)/maximum release] × 100.

RESULTS

Mice were initially injected with FAA at a dose of 330 mg/kg, and splenic NK activity after 12 h was compared with that of control mice and of mice treated 24 h previously with poly I : C (100 μg/mouse). The cytotoxicity measured against YAC-1 targets in FAA-treated mice was significantly above control levels, although not quite as high as with the poly I : C-treated positive controls (Table 1). Lysis of P815 targets was not observed in any group. The ester of FAA, NSC 293 015, was also tested and found to induce NK activity (result not shown).

We next tested the time and dose dependence for induction of NK activity by FAA. Cytotoxicity against YAC-1 targets were measured in spleen cells from mice given FAA (330 mg/kg) at different times before sacrifice. Mice which had been treated 1–2 h earlier contained only control levels of NK activity, but after 4 h the NK levels were significantly increased (Table 2). Highest NK activity was observed after 24 h, where FAA was found to be as potent as poly I : C in boosting NK activity. Combinations of FAA and poly I : C were only slightly more effective than either alone in the

Table 3. Augmentation of NK activity with different doses of FAA

Dose of FAA* (mg/kg)	% Cytotoxicity ± S.E. 200 : 1	100 : 1
330	21.59 ± 0.94	14.52 ± 0.61
220	44.51 ± 0.77	36.18 ± 0.78
150	29.55 ± 0.93	22.55 ± 1.01
100	30.94 ± 1.84	22.35 ± 1.64
45	22.34 ± 1.22	15.89 ± 0.62
0 (control)	16.68 ± 0.63	12.18 ± 0.55

*FAA was given 24 h i.p. and spleen cells were assayed for activity against YAC-1 targets.
*Effector to target cell ratio.

induction of NK activity (Table 2). When assayed at a constant time (24 h) augmentation of NK activity was obtained over a wide range of doses of FAA (Table 3). Highest levels of NK activity were reached with a dose of 220 mg/kg.

NK cells have been shown to be sensitive to treatment with anti-asialo GM-1 [7]. To provide further evidence that the increased cytotoxicity induced by FAA was NK-mediated, mice were pretreated with anti-asialo GM-1 antibody. Table 2 shows that after administration of antibody, no cytolysis of YAC-1 targets could be induced in antibody-treated mice with either FAA or poly I : C.

DISCUSSION

NK cells are a heterogeneous population of effector cells characterized by their common ability to lyse *in vitro* certain susceptible tumour targets without prior sensitization [8] and there is persuasive evidence that the endogenous population of NK cells represent a primary line of defence against tumour growth and metastasis *in vivo* [9]. We have shown that NK activity is elevated as early as 4 h after administration of FAA. The rapid induction of NK activity by FAA suggests a direct action of the drug, although it is possible that a metabolite of the drug could also be active.

It is known that poly I : C augments NK levels via the induction of interferons [8]. The rates of induction of NK activity by FAA and poly I : C are very similar (Table 2) but we have yet to determine whether FAA also works by inducing interferons, or whether it affects NK cells directly. The therapeutic antitumour effects of another group of cytotoxic agents, the 5-halopyrimidones, is also thought to involve the induction of interferon [10] and the augmentation of NK activity [11]. We are currently examining the ability of FAA to induce NK activity *in vitro*, to facilitate the study of its mode of action.

The mechanism of action of FAA on solid tumours is not yet known. We have shown here that a dose of 330 mg/kg, the kinetics of induction of NK activity by FAA approximate the kinetics of induction of haemorrhagic necrosis of the colon 38 tumour [5]. However, we have yet to establish whether NK cells are involved in FAA-induced regression. Pre-injection of anti-asialo GM-1 antibody, under the same conditions as described in Table 2, does not prevent the induction of necrosis of the colon 38 tumour by FAA (results not shown). Experiments to evaluate the role of NK and other immune mechanisms in FAA induced regression of solid tumours are in progress.

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