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Role of T Cells and Tumour Necrosis Factor in Antitumour Activity and Toxicity of Flavone Acetic Acid

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To investigate the importance of natural killer (NK) and T cells in the inhibition of tumour growth by flavone acetic acid (FAA), colon 26 murine carcinoma was grafted subcutaneously in euthymic and athymic mice. FAA was active in euthymic but not in athymic mice (ratio between tumour weight in treated vs. control animals [T/C %], 27% and 92%, respectively). NK cell activity was increased in both mouse strains, indicating a lack of major involvement of this lymphocyte population in FAA efficacy. In euthymic mice tumour-specific T cells were activated, and after *in vivo* depletion of lymphocyte subpopulations (L3T4 and Lyt2), tumour inhibition by FAA was abrogated (T/C %, 88%). Antitumour efficacy of FAA was also reduced when the treatment was followed by injection of antitumour necrosis factor alpha (TNFα) antibodies. FAA toxicity depended on tumour weight at the time of treatment: 200 mg/kg caused 0 and 100% mortality in mice bearing tumour nodules under 50 and over 300 mg, respectively. When anti-TNFα antibodies were given after FAA treatment, the toxicity was greatly reduced (3/14 mice died compared with 10/15).
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INTRODUCTION

FLAVONE ACETIC ACID (FAA) is one of the most active drugs against murine transplantable subcutaneous murine tumours [1, 2]. However, disappointing results were obtained in phase II clinical trials [3, 4]. The reasons for the discrepancy are unknown and may be due to different mechanisms of action in mouse and man. The mechanism of action of FAA against murine tumours contrasts in several ways with that of conventional cytotoxic drugs, and host-mediated factors appear to be involved, which places FAA in the category of biological response modifiers. Natural killer (NK) cell activity is activated [5, 8] and interferons [6] and tumour haemorrhagic necrosis are induced in mice treated with FAA [8–10]. Antitumour T cell response was activated after administration of FAA plus interleukin 2 (IL-2) [7], whereas cytotoxic tumour infiltrating lymphocyte activity was reduced following FAA treatment [11]. However, a link between immunomodulation induced by FAA and its antitumour activity has yet to be confirmed [12].

The aim of our study was to investigate the importance of T and NK cells in FAA antitumour activity by comparing efficacy against a murine tumour growing subcutaneously in euthymic and athymic nude mice. Colon 26 (C-26) tumour was chosen since FAA reduces tumour growth and induces haemorrhagic necrosis of this murine model [9]. The role of T cells in tumour growth inhibition in BALB/c euthymic mice was further evaluated *in vivo* by depleting T lymphocytes with specific monoclonal antibodies (MAb). In addition we investigated the importance of tumour necrosis factor (TNF) in the antitumour and toxic effects of FAA.

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MATERIALS AND METHODS

Mice

Euthymic BALB/c and athymic CD1 (nu/nu) mice from Charles River Laboratories were used when 8–10-weeks-old. Sterilised cages, bedding, food and water were used.

Drugs

FAA, supplied by the Division of Cancer Treatment, National Cancer Institute (Bethesda), was dissolved in 5% $NaHCO_3$ and delivered intravenously at 10 ml/kg.

Tumour lines

C-26 adenocarcinoma [13] was maintained *in vivo* by subcutaneous transplantation of tumour fragments in syngeneic mice. The C-26 cell line used in the *in vitro* experiments was established from a subcutaneous nodule and maintained as described [14]. Other tumour cell lines used were the B16 melanoma C57BL/6 strain, and the NK-sensitive YAC lymphoma, both maintained in continuous *in vitro* cultures. The cell lines were shown to be mycoplasma-free by periodic electron microscopy.

Assessment of antitumour activity

C-26 tumour fragments were grafted subcutaneously by a 13 G trocar in the left flank of euthymic or athymic mice. Tumour growth was followed by biweekly caliper measurement of longest (length) and shortest (width) diameters. Tumour weight in mg was calculated by width^2 × length ÷ 2 [15]. Median survival time was calculated for each group. Effects of FAA treatment were expressed as T/C % i.e. the ratio between values in treated and control mice times 100. Tumour-bearing mice dying of toxicity were always included. When not otherwise indicated, two-tailed *t* tests were used.

Cell-mediated cytotoxicity

NK and T cell cytotoxicity was assayed in a standard 4 h ^{51}Cr-release assay [14]. To assess NK activity, fresh peripheral

blood lymphocytes (PBL) and spleen lymphocytes (SPC) were separated on Ficoll gradients (Lympholite M, Cederlane, Ontario) and tested against the YAC target. Mixed lymphocyte tumour cell cultures were set up with spleen cells and C-26 tumour cells irradiated with 15 000 rad [14]. Each value in the tables represents the average of three replicates; standard deviation never exceeded 10%.

MAbs

Hybridomas GK1.5 and 2.43 producing rat IgG_{2b} against the L3T4 (CD4) and Lyt2 (CD8) T cell antigens, respectively, were obtained from American Type Culture Collection, Rockville. For T cell depletion, C-26 tumour-bearing mice were given 0.1 ml of MAb ascitic fluid intraperitoneally twice with a 24 h interval between injections. This procedure induces long-term depletion of T cells *in vivo* [16]. We found near complete depletion (over 95%) of each specific T cell subpopulation by flow cytometry in spleen and lymph node cells 5 days after treatment. Moreover, depletion of either L3T4 or Lyt2 cells (or both) resulted in failure to generate cytotoxic T lymphocytes to allogeneic target following *in vitro* sensitisation (data not shown).

Rabbit anti-murine TNFα antiserum (Genzyme, Boston) was injected intravenously at 5×10^4 neutralising units, a dose which inhibits endotoxin toxicity [17], 30 min after FAA.

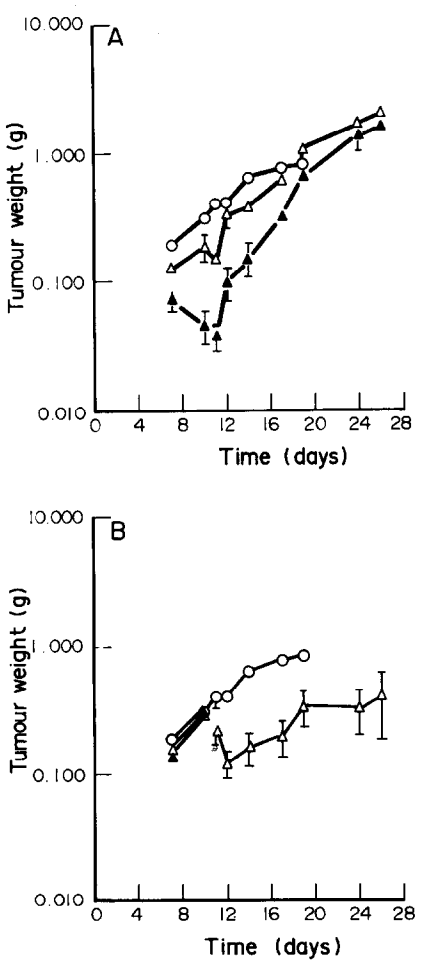

Fig. 1. Efficacy of FAA against early (A) or advanced (B) subcutaneous C-26 tumour. ○ = control, △ = FAA 150 mg/kg and ▲ = FAA 200 mg/kg. Mean (S.E.) Arrows = treatment days.

Table 1. *Effects of FAA on* C-26 *tumour-bearing BALB/c mice*

Dose (mg/kg)	Days of treatment*	T/C % Mean tumour weight†	T/C % Median survival	Toxicity (dead/total)
150	3	52%‡	126%	0/15
200	3	15%§	135%	0/15
150	10	26%§	117%	4/13
200	10	—	43%‖	12/12

* After tumour inoculum.
† 1 week after last treatment.
Significant differences vs. controls: ‡ $P < 0.05$, § $P < 0.01$ and ‖ $P < 0.0001$.

RESULTS

FAA antitumour effects

BALB/c mice bearing early (day 3) or advanced (day 10) tumours were treated with two FAA doses, 150 or 200 mg/kg. The original schedule included one treatment every 4 days for a total of three treatments, but 200 mg/kg proved unexpectedly toxic to mice bearing advanced tumours and therefore the number of treatments was reduced. Thus, early tumours were treated twice and the advanced ones once. Growth curves of tumours in control and treated mice are shown in Fig. 1. FAA (150 mg/kg) was more efficacious in inhibiting tumour growth of advanced (panel B) than of early tumours (panel A). The effect (T/C %) of FAA on mean tumour weight 1 week after the last treatment was 26% and 52% in advanced and early tumours respectively (Table 1). The higher dose (200 mg/kg) of FAA was tolerated only in early tumour-bearing mice and was very effective (T/C = 15%). Survival was marginally affected in all groups except in mice bearing advanced tumours treated with 200 mg/kg (Table 1). In this group all mice died of toxicity within 24 h from treatment.

To evaluate the role of T cells in tumour inhibition by FAA, C-26 fragments were grafted in BALB/c and in genetically athymic Swiss nude mice. Tumour growth curves were comparable in the controls of the two recipient mice strains. FAA (200 mg/kg at day 7) inhibited tumour growth in the euthymic BALB/c mice but not in athymic mice (Table 2). Athymic control mice survived longer than the immunocompetent mice. In the treated mice, survival was unaffected in nude mice and decreased in the euthymic mice due to early deaths from toxicity

Table 2. *Effects of FAA on* C-26 *tumour-bearing BALB/c and Swiss nu/nu mice**

Mice	T/C % Mean tumour weight†	T/C % Median survival	Toxicity (dead/total)
BALB/c	27%‡	53%	4/9
Swiss nu/nu	92%	100%	0/5

* In BALB/c and Swiss control mice, mean tumour weight was 1117 (S.E. 147) and 1261 (267) mg and median survival was 15 and 31 days, respectively.
† 1 week after the drug treatment.
‡ $P < 0.01$ compared with controls.

Table 3. Toxicity of FAA (200 mg/kg) in mice bearing C-26 tumours

Mice	Mean tumour weight when dosed (mg)	Toxicity (dead/total)
BALB/c	< 50	0/15
	> 150	4/9
	> 300	12/12
Swiss nu/nu	> 300	0/5

Table 4. Enhancement of NK activity after treatment with FAA in BALB/c and Swiss nu/nu mice bearing C-26 tumours

Mice	Days after treatment*	Cytotoxicity (%) on YAC target†	
		Peripheral blood lymphocytes	Spleen lymphocytes
BALB/c	Controls	8	2
	1	12	43
	3	15	16
	7	15	20
Swiss nu/nu	Controls	9	11
	1	10	32
	3	13	31
	7	17	29

* FAA (200 mg/kg) was given 7 days after tumour inoculation.
† Effector: target ratio 100:1.

(Table 2). The same dose of FAA in BALB/c mice was differently toxic depending on tumour weight, whereas the drug was tolerated in athymic Swiss mice bearing large tumours (Table 3). Haemorrhagic tumour necrosis was observed in euthymic and athymic mice, treated with FAA, independently of tumour weight.

NK and T cell activation

NK cytotoxic activity in peripheral blood and spleen lymphocytes was assessed at different times after FAA administration (Table 4). NK activity was similarly increased in both athymic and euthymic mice, especially in the spleen. Differences in NK activity of spleen lymphocytes between conventional and nude mice were marginal and considered biologically irrelevant.

Cytotoxic activity specific for C-26 target cells was present in cultures of lymphocytes obtained 3 and 7 days after FAA treatment, indicating that T cell activation occurred *in vivo* (Table 5).

Table 5. Activation of specific cytotoxic lymphocytes in BALB/c mice bearing C-26 tumours after FAA treatment (200 mg/kg)

Days after FAA treatment	Cytotoxicity (%)*	
	C-26	B16
Controls	3	4
1	0	1
3	24	3
7	27	1

* Effector: target ratio 50:1 after *in vitro* stimulation (see Methods).

*Table 6. Effect of FAA on C-26 tumour in BALB/c mice previously depleted of T cell subpopulations with specific MAbs**

	No. of mice	Mean tumour weight (T/C %)†
Controls	12	37
MAb against:		
Lyt2	6	56
L3T4	7	98
Lyt2 + L3T4	7	88‡

* FAA 200 mg/kg 9 days after tumour inoculation.
† 1 week after treatment. Mean tumour weight of control was 742 (101) mg.
‡ $P < 0.05$ compared with controls.

Role of T cells and TNF in FAA induced tumour regression and toxicity

When FAA was delivered to tumour-bearing mice depleted of Lyt2 lymphocytes, tumour regression was similar to that achieved in non-depleted controls (Table 6). In contrast, in mice depleted of L3T4 cells or depleted of both Lyt2 and L3T4 subsets, FAA activity was inhibited (significant only in one group, possibly due to the small number of mice). Therefore, the L3T4 lymphocyte subpopulation appears to be involved in FAA induced regression of C-26 tumour in immunocompetent mice.

Only 3 out of 14 mice died of toxicity in the group receiving FAA plus anti-TNFα antibodies, whereas 10/15 died in the group receiving only FAA (Table 7). Administration of anti-TNFα serum decreased FAA antitumour activity compared to that in control FAA treated mice. Survival time was decreased in FAA-treated mice due to drug toxicity, whereas it was increased in the group receiving anti-TNFα antibodies. Statistical significance was not achieved compared with control mice, possibly due to the fact that mice dying of toxicity all died 24 h after FAA treatment, whereas mice surviving toxicity died later and the interval of deaths for control mice lay in the interval for treated mice.

DISCUSSION

This study emphasised an important role of T cells in FAA's antitumour activity against subcutaneously growing C-26

*Table 7. Effect of anti-TNFα serum on FAA toxicity and antitumour activity in C-26 tumour-bearing BALB/c mice**

Anti-TNFα serum	Toxicity (dead/total)†	T/C %‡	
		Mean tumour weight	Median survival
−	10/15	37% §	48%
+	3/14	71%	145%

* FAA 200 mg/kg was delivered when mean tumour weight was over 300 mg.
† Within 24 h of FAA treatment: χ^2 test, $P < 0.05$.
‡ 7 days after drug treatment for mean tumour weight. In control mice mean tumour weight was 780 (±81) mg and median survival was 21 days.
§ $P < 0.01$ compared with controls.

tumour since an antitumour effect was evident in euthymic but not in athymic mice. Moreover, FAA induced tumour regression was blocked in BALB/c mice depleted of L3T4 cells or of both L3T4 and Lyt2 T cells. The critical role of the L3T4 lymphocyte subpopulation suggests that the antitumour effect of FAA may be due to the release of cytokines. In accord with this hypothesis, the induction by FAA of TNFα, interferons alpha and gamma and IL-1 gene expression in lymphocytes of BALB/c mice parallels the antitumour efficacy of this drug [18]. In our study, FAA treated mice displayed activation of Lyt2 splenic cytotoxic T lymphocytes, but this cell population does not appear to be critical for FAA induced tumour regression since mice specifically depleted did not show differences in tumour response compared with undepleted controls. Similar conclusions are suggested by the reduction of cytotoxic activity of tumour infiltrating lymphocytes isolated after FAA treatment [11].

Enhanced NK activity following FAA treatment has been documented in healthy and tumour-bearing mice. In our study, even though both BALB/c and nude athymic mice displayed enhanced NK activity in peripheral blood lymphocytes and spleen cells after FAA treatment, an antitumour effect was observed only in BALB/c mice. Moreover, BALB/c mice treated repeatedly with FAA before tumour grafting did not show any difference in latency times or growth curves of the tumour compared with untreated controls (data not shown). The increase in NK cell activity is therefore not a critical component in FAA efficacy against subcutaneously growing tumours. However, we cannot exclude a role for NK cells in FAA efficacy against tumours growing at other sites, such as liver colonies which showed high sensitivity to FAA even in a human tumour xenografted in nude mice [19].

A TNF-like behaviour for FAA has also been proposed, based on the observation of rapidly induced haemorrhagic tumour necrosis after treatment with the drug. However, in our study no correlation was found between tumour response, achieved only in BALB/c mice, and the presence of haemorrhagic tumour necrosis, achieved in both athymic and euthymic mice. Moreover, in tumour-bearing BALB/c mice treated with anti-TNFα antibodies after FAA, in which tumour growth was inhibited (T/C % for mean tumour weight of 71%), haemorrhagic tumour necrosis was occasionally observed. However, TNF-mediated mechanisms play a role in FAA biological effects. In fact, a reduction in FAA antitumour activity was observed in mice depleted of TNFα as well as a striking decrease in lethal toxicity. Increased toxicity of FAA in large tumour-bearing mice has only occasionally been described in other murine models [20–21]. C-26 tumour induces a cachexia [22], and this effect is likely to be mediated by the production of cachectin (i.e. TNF), by the tumour itself or through host-mediated mechanisms [23]. If so, serum TNF levels in mice bearing a large C-26 tumour will be higher than in mice bearing a small C-26 tumour and TNF stimulation induced by FAA injection might give rise to a lethally high TNF level. This hypothesis may be supported by the finding that FAA and TNF induce indistinguishable histological effects (tumour necrosis in subcutaneous colon-38 tumour) [24]. Moreover, FAA toxicity in mice bearing bulky (over 300 mg) C-26 tumours cannot be ascribed to the performance status since in the same experimental conditions the toxicity of conventional cytotoxic drugs such as cisplatin or doxorubicin was not increased (data not shown). The increase in survival time in mice treated with anti-TNFα antibodies might be due to an improvement in general condition. Therefore, host-mediated effects played an important role either in C-26 tumour-induced cachexia or in FAA toxicity, since these two effects were not present in the tumour-bearing athymic mice. A T-cell mediated mechanism has been demonstrated for the antitumour effects of *in vivo* treatment with TNF α [25, 26].

The role of macrophages in FAA antitumour activity was not investigated in our study. Enhancement of tumoricidal activity by FAA [27] and lack of correlation between macrophages and haemorrhagic tumour necrosis [28] have been reported. Their relevance has still to be clarified.

For subcutaneously growing tumours, as well as host-mediated effects, other mechanisms have been suggested for FAA activity, possibly due to an indirect antitumour activity. FAA has strong effects on tumour blood flow [20, 29–31], as a consequence of intravascular coagulation causing vascular occlusion and ischaemic tumour cell death [32].

A combination of immunomodulation and indirect cytotoxic mechanisms is probably responsible for the unique spectrum of activity of FAA in subcutaneously growing murine tumours.

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Effects of Spontaneous Physical Exercise on Experimental Cancer Anorexia and Cachexia

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The aim of this study was to evaluate whether spontaneous physical exercise can modify cancer anorexia and cachexia in tumour-bearing rats. Two transplantable experimental tumours were evaluated. Tumour-bearing Wistar Furth rats fed *ad libitum* and with free access to a running wheel had a delayed onset of anorexia compared with their non-exercised tumour-bearing controls, retained normal behaviour and were able to run the same daily distance as non-tumour controls until the onset of cachexia. Exercise resulted in a decreased carcass wet weight and lipid stores but in an increased carcass dry weight in the tumour-bearing animals. Despite increased food intake, physical exercise resulted in a reduced final tumour weight without any change in water content. Skeletal and cardiac muscle tissue did not show any difference in water content but there was an increased RNA/protein quotient in the exercising tumour-bearing animals. Thus the deleterious alterations induced by the malignancy on tumour host metabolism are not inevitable but can be modified by spontaneous physical exercise.
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INTRODUCTION

EARLY EXPERIMENTAL studies showed beneficial effects of physical exercise on tumour growth [1–3] but effects on tumour host metabolism were not evaluated. It is not known whether the effects of physical exercise on tumour host metabolism are primary metabolic events, or secondary to an effect on tumour growth itself. Superimposed on these effects is the changed motor activity that has been noted in experimental tumour models [4]. Experimental studies of the effect of physical exercise on metabolism often include forced exercise to standardise the experiment and the amount of activity [5]. This might increase stress with subsequent neuroendocrine changes compared with spontaneous activity. Promotion of experimental tumour growth as a result of stress has been described [6, 7], as have adverse effects on skeletal muscle metabolism in man [8] and in rodents [9, 10].

In view of the possibly negative side-effects of forced physical activity on the metabolism of the tumour host and/or the tumour

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