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Reduction of Cytotoxic Effector Cell Activity in Colon 38 Tumours Following Treatment with Flavone Acetic Acid*

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Abstract—*Host cells have been implicated as being involved in the antitumour effects of flavone acetic acid (FAA), an agent with selectivity towards solid tumours which is currently undergoing clinical trial. To determine whether tumour-associated host cells are affected by FAA treatment, tumour-infiltrating leukocytes (TIL) were isolated from subcutaneous Colon 38 tumours, which are known to be sensitive to FAA. 1–2 × 10^5 TIL were isolated per gram of tumour, comprising mainly small lymphocytes and macrophages. Spontaneous activity against YAC-1 and P815 tumour targets was tested in a 4 h ^{51}Cr-release assay for lymphoid cytotoxic effector cells. High levels of activity were exhibited by TIL against both P815, which is resistant to natural killer (NK) cells, and to NK-sensitive YAC-1 cells. In contrast, splenic cell populations contained only NK cell activity. Within 1 h of intraperitoneal administration of FAA (330 mg/kg) the cytotoxic effector cell activity of the TIL population was dramatically depressed, remaining low during the time in which extensive tumour necrosis became evident. In contrast, splenic NK activity was unchanged at 1 h and elevated at 4 h. The decrease in lymphoid killer activity of the TIL population following treatment argues against the primary involvement of these effector cells in mediating the antitumour action of FAA.*

INTRODUCTION

FLAVONE ACETIC ACID (FAA), a synthetic flavonoid [1] now undergoing clinical trial [2], has been found to have high cytotoxic activity against a broad spectrum of experimental slow growing solid tumours but limited activity against leukaemias [3, 4]. The mode of action of FAA in mice is unusual in that it does not appear to act directly on tumour cells as do most clinical antitumour agents [5]. Several observations such as the induction of interferon production following *in vivo* administration [6] and the systemic elevation of natural killer (NK) activity in both normal [7, 8] and tumour-bearing mice [9, 10] place FAA in the category of biological response modifiers (BRM). These results raise the possibility that FAA mediates its antitumour effects through activation of host cytotoxic mechanisms.

Most tumours contain an infiltration of host lymphoreticular cells which can be regarded as a manifestation of immunological recognition of the tumour by the host [11]. If host cells are involved in FAA-induced tumour necrosis then one might expect tumour-infiltrating host cells to be involved. The Colon 38 tumour is highly susceptible to FAA and undergoes rapid and extensive haemorrhagic necrosis upon *in vivo* administration of FAA [12]. We have therefore attempted to characterize the tumour-infiltrating leukocytes (TIL) in this tumour by measuring their levels of cytotoxic effector activity before and after FAA treatment.

MATERIALS AND METHODS

Materials

FAA, obtained from the National Cancer Institute, U.S.A., through the courtesy of Dr K.D. Paull, was dissolved in 5% sodium bicarbonate (33 mg/ml). Antibody to asialo-GM-1 glycosphingolipid (anti-GM-1) was purchased from Wako Pure Chemicals Industries, Osaka, Japan. 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.

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Mice

$C_{57}BL/10J$ (B10), $C_{57}BL/6J$ and $(C_{57}BL/6J \times C_3H/HeN)F_1$ (BCF_1) mice (stocks originating from Jackson Laboratory, Bar Harbor, U.S.A.) between 8 and 12 weeks of age were bred in the laboratory animal facility 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 subcutaneously (s.c.) in $C_{57}BL/6J$ mice and grown for experiments in BCF_1 or B10 mice. Tumours were grown to a diameter of approx. 10 mm in carrier mice, removed surgically and cut into 1 mm^3 fragments for implantation in recipient mice anaesthetized by intraperitoneal (i.p.) injection of pentobarbital (90 mg/kg; dissolved in 0.2 ml physiological saline). P815 mastocytoma and YAC-1 lymphoma cells were obtained from Dr J. Marbrook, Department of Immunobiology, University of Auckland Medical School, and were maintained in culture as previously described [7].

Preparation of spleen cells and TIL

Mice were killed by cervical dislocation and spleens were removed, teased out into culture medium, aspirated to give a single cell suspension, transferred into a tube and allowed to settle for 5 min to remove any clumps. Viable white cells, distinguished by their ability to exclude eosin red, were counted in a haemocytometer.

Colon 38 tumours were removed, minced and disaggregated by extrusion through a nytex mesh. The cell suspension was placed in a tube, debris allowed to settle for 5 min and the cells remaining in suspension transferred into another tube. The cells were harvested by centrifugation, resuspended in fresh medium and layered over Ficoll (specific gravity 1.077 g/ml; Immunochemicals Ltd., Auckland, New Zealand). After centrifugation (200 ***g***, 8 min) the cells in the leukocyte band removed washed and counted. Ficoll of density 1.077 g/ml was used since Ficoll of density 1.114 g/ml, which is normally used to retain all leukocytes, was found to result in heavy contamination of leukocytes with tumour cells and debris.

Cytotoxicity assays

Cytotoxic effector cell activity was measured using a 4 h ^{51}Cr-release assay [13]. Lysis over a range of effector to target (E:T) ratios 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 (experimental release) was measured by removing 0.1 ml of the supernatant and counting in a gamma counter (LKB Wallac 1270 Rackgamma 11, Wallac, Finland). Radioactivity in the supernatants of six wells receiving target cells alone (spontaneous release) and in six wells containing resuspended target cells (total radioactivity) was also measured. The percentage lysis was calculated as [(experimental release minus spontaneous release)/(total radioactivity)] × 100. Assays with spleen effector cells were carried out in quadruplicate while assays containing TIL, because of the low numbers of leukocytes available, were carried out singly or in duplicate. The variability between replicate samples was such that an assay value of 5% lysis was statistically significant ($P < 0.01$). The standard deviation of replicate assays in the range 5–50% lysis was typically ± 10% (range ± 4 to ± 20%).

RESULTS

Characterization of TIL in Colon 38 tumours

Subcutaneous Colon 38 tumours were removed, disaggregated and the leukocytes separated by Ficoll density fractionation. Approximately $1–2 \times 10^5$ viable leukocytes were isolated from each gram of tumour tissue. Cells were stained with Leishman's stain and their morphology examined. The majority of the host cells were shown to be small lymphocytes and macrophages, although the proportion of lymphocytes to macrophages varied in different experiments. The majority of preparations contained a higher proportion of lymphocytes than macrophages. Granulocytes comprised less than 1% of the TIL and most of the granulocytes were basophils; neutrophils were seldom seen.

Lytic activity of cytotoxic lymphoid effector cells in TIL

TIL were isolated as above from s.c. Colon 38 tumours in B10 or BCF_1 mice and assayed for lysis of YAC-1 and P815 targets in a 4 h ^{51}Cr-release assay for cytotoxic lymphocytes. Because of the small number of cells obtained, cells were pooled from five to 10 mice for each experiment. YAC-1 cells were used as targets for NK cells while P815 cells, which are not susceptible to NK-mediated killing [14], served to detect the presence of other types of activated killer cells such as the lymphokine activated killer (LAK) cells. In several experiments with either B10 or BCF_1 mice, TIL demonstrated high levels of activity against both YAC-1 and P815 targets; the results of one experiment are shown in Fig. 1. Spleen cells from the same animals were

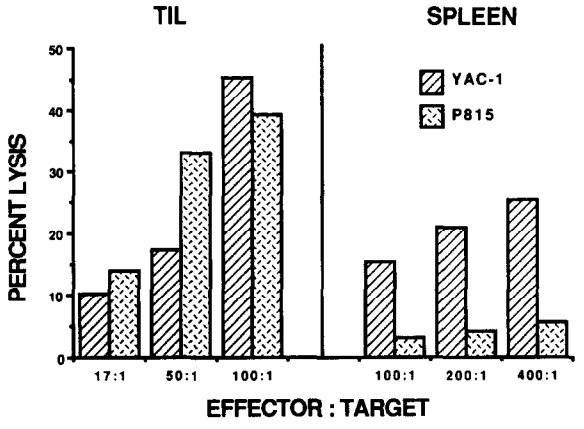

Fig. 1. Cytotoxicity against YAC-1 and P815 targets in TIL and spleen cells. Spleen cells and TIL isolated from s.c. Colon 38 tumours from BCF_1 mice were assayed against YAC-1 (hatched bars) and P815 (dotted bars) at varying E:T cell ratios.

found to be highly active against YAC-1 targets (Fig. 1) but lysed less than 5% of P815 targets at all E:T cell ratios tested. The amount of killer cell activity in TIL was also much higher than that in the spleen (Fig. 1). Spleen cells at an E:T ratio of 200:1 lysed 20% of YAC-1 targets whereas, with TIL, an E:T ratio of only 50:1 was required for similar levels of lysis.

Depression of cytotoxic activity in TIL by anti-GM-1

NK cells in mice express on their surface the glycosphingolipid asialo GM-1, and administration of antibody to this antigen has been shown to deplete NK activity in the spleen [7, 10, 15]. The effect of this antibody on the TIL population was examined. TIL and spleen cells were isolated from s.c. Colon 38 tumours from control mice and mice treated 3 days previously with anti-GM-1 (50 μg/mouse). Cytolytic activity of TIL (E:T ratio 100:1) was found to be reduced for both YAC-1 (10% lysis compared to control of 29%) and P815 (10% lysis compared to control of 27%) targets. Cytolytic activity of spleen cells against YAC-1 targets (E:T ratio 200:1) was also reduced (7% lysis compared to control of 26%).

Killer cell activity in TIL after FAA treatment

The above results indicate that the TIL population contain high levels of killer cell activity. To examine the possible role of these killer cells in FAA-induced tumour necrosis, we measured activity in TIL at different times after FAA treatment. Groups of five to seven B10 host mice, each with s.c. Colon 38 tumours of size 1–2 g, and either untreated or treated with FAA (330 mg/kg i.p.) were sacrificed 1, 2, 3 or 4 h later. Longer times were not used because the soft and necrotic nature of the tumour after 4 h made the isolation of leukocytes difficult. Tumours and spleen were removed, pooled and the lytic activity of TIL and spleen cells was measured as described above using both YAC-1 and P815 targets at several E:T cell ratios. Because of the small numbers of leukocytes present in tumours, each time point was determined in a different experiment with a minimum of two experiments per time point.

Similar numbers of TIL were obtained from FAA-treated and -untreated tumours, suggesting that no influx of TIL occurred after FAA treatment. All samples lysed both YAC-1 and P815 targets. However, at all time points measured, the cytotoxic activity of TIL from FAA-treated mice against both targets was decreased as compared to that of untreated mice. As early as 1 h after FAA treatment, the lytic activity of TIL from FAA-treated mice was significantly lower than that of control mice over four independent experiments ($P < 0.05$). The response of spleen cells from tumour-bearing mice was the same as that reported previously for normal mice [7, 10]; no significant activity was detected against P815 targets while lysis of YAC-1 targets was unchanged 1 h after but augmented 4 h after FAA treatment. Representative data at E:T ratios of 100:1 for TIL and 200:1 for spleen cells are shown in Fig. 2.

DISCUSSION

TIL isolated from Colon 38 tumours comprise less than 1% of the total tumour and consist predominantly of small lymphocytes and macrophages with very few granulocytes. More lymphocytes than macrophages were found in most preparations, but this bias could result from the fractionation procedure since the density of Ficoll used to reduce contamination of tumour cells and debris might favour the enrichment of lymphocytes. Nevertheless, it is evident that macrophages and lymphocytes are the main types of host leukocytes infiltrating the Colon 38 tumour, consistent with the findings by many groups of infiltrating host cells in other tumours [11, 16–21]. Some tumours are infiltrated primarily by lymphocytes and minor proportions of macrophages [17, 18] while others contain a higher content of macrophages [18, 19].

The Colon 38 tumour has a much lower concentration of leukocytes than some other tumours, where up to 60% of the total cells are leukocytes [17, 19]. Some groups have suggested that a low host cell content is correlated with a high metastatic potential [20]. We have not been able to demonstrate metastasis of the Colon 38 tumour following s.c. or i.v. inoculation (unpublished results) although it grows in the liver after intrasplenic inoculation [12]. Other groups find no correlation between metastatic ability and TIL content [19, 21] and the role of TIL in the control of tumour growth and metastasis thus remains uncertain.

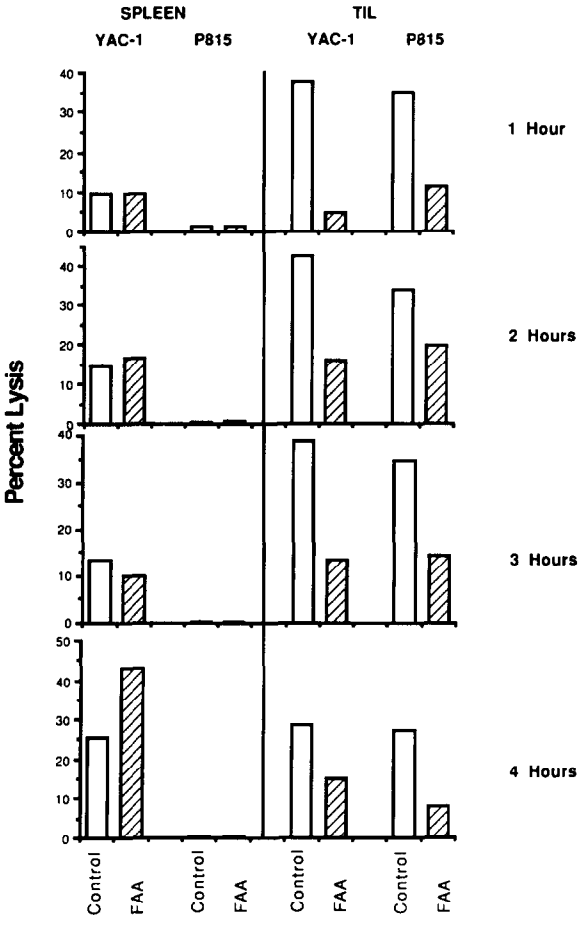

Fig. 2. Cytotoxic activity in spleen and TIL after FAA treatment. B10 mice with s.c. Colon 38 tumours were treated with FAA (330 mg/kg i.p.). Spleens and tumours were removed at the indicated times and the cytotoxic activity in the spleen cell and TIL populations measured against YAC-1 and P815 targets. Results are shown for 200:1 spleen E:T cell ratio and 100:1 TIL E:T cell ratio for FAA-treated tumour-bearers (hatched bars) and untreated tumour-bearers (open bars).

Various groups have studied the presence of NK cells in tumours and have concluded that their concentration is generally very low compared to that in peripheral blood [16, 17] for reasons which are still unclear [17, 22]. We have found that TIL from Colon 38 tumours growing in either $C_{57}BL/10J$ mice (congenic with the strain of origin) or hybrid mice (BCF_1) contain much higher levels of cytolytic activity against YAC-1 targets than do spleen cells from the same animal measured at the same E:T ratio (Fig. 1). TIL also contain effector cells which lyse NK-resistant targets, in contrast to spleen cells which contain only NK activity (Fig. 1).

Spleen cells can develop activity against NK-resistant targets after culture with interleukin 2 through the production of LAK cells, and this process is enhanced by prior *in vivo* treatment with FAA [10]. Since LAK cells appear to be generated from NK cells in the presence of interleukin 2, NK and LAK cell may represent cells of the same lineage at different stages of activation in response to different environments [23, 24]. It is possible that within the microenvironment of the tumour, higher levels of lymphokines are produced, leading to lymphoid effector cells with the cytolytic characteristics of LAK cells rather than NK cells.

Cytolytic activity to both YAC-1 and P815 targets was reduced by prior treatment with anti-GM-1, suggesting that at least some of these cells either have asialo-GM-1 glycosphingolipid on their surface (in common with NK cells) or are dependent on asialo-GM-1 positive cells for their development.

NK activity in the spleen in augmented after FAA administration [7, 8, 10] (Fig. 2) and the time course of NK augmentation parallels the time course of induction of tumour necrosis [7, 12], raising the possibility that the antitumour effects of FAA are mediated by the elevation of NK activity. However, FAA induces haemorrhagic necrosis of NK-resistant tumours [10], arguing against the primary involvement of NK cells in this process. NK activity can also be induced by treatments which do not cause necrosis of Colon 38 tumours [10]. Moreover, the present results show that a decrease rather than an increase of cytolytic activity in the TIL population accompanies FAA-induced tumour necrosis (Fig. 2). FAA induces tumour necrosis equally well in normal mice and in mice previously treated with anti-GM-1, a procedure which reduces cytolytic activity of both splenic NK cells [7, 10] and TIL (this work). Taken together, these observations suggest a lack of correlation between NK induction and tumour necrosis. Thus, NK cells do not appear, at least in the Colon 38 tumour, to be the prime mediators of FAA antitumour effects, even though they may be important for long-term recovery and prevention of recurrence of tumours [9].

The reason for the rapid loss of killer cell function in TIL after FAA administration is unclear. It is unlikely to be due to an efflux of effector cells since the yields of TIL in treated and control tumours are similar. Perhaps, in response to FAA, effector cells are activated and expend their lytic potential during the first hour. NK cells at tumour sites appear to be unable to recycle for multiple lytic events, in contrast to NK cells from other organs [15]. At least in the case of the Lewis lung carcinoma there is little evidence for FAA-induced killing of tumour cells during the first 2 h after treatment; killing is maximal between 4 and 8 h after treatment [5]. On the other hand, changes in tumour blood flow are detectable within 30 min of FAA administration (L.J. Zwi, B.C. Baguley, J.B. Gavin and W.R. Wilson, *J Natl Cancer Inst* 1989, in press). Analysis of such early FAA-induced events in the tumour should provide a better understanding of changes in the TIL population.

The other cell type prevalent in the TIL population which has not yet been discussed is the macrophage. While FAA does not augment NK

activity *in vitro*, the tumoricidal activity of macrophages can be significantly enhanced by exposure to FAA in culture [25]. This result suggests that FAA may act in way on macrophages *in vivo*. Our current experiments are aimed at elucidating the action of FAA on macrophages.

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