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Immunological Effects of Flavone Acetic Acid

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ABSTRACT

Flavone acetic acid (FAA) enhances natural killer and lymphokine-activated killer (LAK) cell activity in mice. We examined the immunological effects of FAA on human blood cells both *in vivo* and *in vitro*. Peripheral blood natural killer and LAK activity and lymphocyte subsets were evaluated in cancer patients after receiving 3-h infusion of FAA at either 8.5 or 10 g/m² with alkalinization. Natural killer cell activity and the number of Leu-19 (CD56) positive cells decreased at 24 h after infusion; significant changes in LAK activity and the number of Leu-1 (CD5), Leu-3 (CD4), Leu-2 (CD8) cells were not observed. Peripheral blood mononuclear cells and peripheral blood lymphocytes collected from healthy volunteers were exposed *in vitro* to FAA, interleukin 2, and FAA plus interleukin 2. FAA, alone or in combination, failed to enhance LAK activity at any time point or concentration from peripheral blood mononuclear cells and peripheral blood lymphocytes. Concentrations of ≥100 μg/ml antagonized the generation of LAK activity from interleukin 2 treated peripheral blood lymphocytes. These data suggest that FAA may not be useful in enhancing immunological responses in humans.

INTRODUCTION

FAA[1] is one of a series of synthetic flavonoids, benzo[γ]-pyrone derivatives, found naturally in all plant cells. Preclinical studies demonstrated activity against tumors refractory to conventional chemotherapeutic agents (1, 2). Although the antitumor mechanism of FAA is not known, evidence suggests that FAA does not act directly on tumor cells (3). Rather, there is evidence that FAA acts by effecting immunomodulation (3). FAA has been shown to augment NK and LAK activity in mice and to synergize with IL-2 in the treatment of BALB/c mice bearing Renca renal carcinoma (4). Preliminary studies in cancer patients have also suggested immunological effects. NK cell activity was significantly increased in 3 of 6 patients receiving FAA at 6.4 g/m² by 3-h infusion in a phase I study (5). In another phase I study, 1 of 3 patients receiving 10 g/m² over 1 h demonstrated increased NK activity (6). Transient increases in NK cells identified with MAB, Leu-19 (CD56), have also been reported with FAA (6). We investigated the effects of FAA on NK and LAK activity and on lymphocyte subsets in cancer patients receiving FAA and also investigated the effects of FAA on LAK generation *in vitro*.

MATERIALS AND METHODS

Patient Studies. Eight cancer patients receiving multiple infusions of FAA as part of a phase I study were investigated (Table 1). The protocol was reviewed and approved by The Ohio State University Institutional Review Board, and all patients provided written, informed consent. FAA was administered as a 3-h infusion at 8.5 g/m² (patients 1–3) and at 10 g/m² (patients 4–8) every 21 to 28 days for up to 5 courses. Sodium bicarbonate was administered throughout the FAA infusion to maintain the urine pH at >7.0. Peripheral blood was drawn into preservative-free heparin prior to FAA infusion and at various time points after completion of the infusion. PBMC were separated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation. Monocytes were depleted from PBMC using phenylalanine methyl ester to yield PBL (7). Cytotoxicity was determined using freshly separated cells. Cells were cryopreserved in a controlled-rate freezer at −70°C for analysis of lymphocyte subsets at the completion of treatment cycles.

Cytotoxicity Assays. Cytotoxicity was determined using standard 4-h ^{51}Cr release assays as described previously (7). NK cell activity was determined using K-562 cells as targets, and LAK activity by using NK-resistant Raji cells. "Il-2 augmented NK activity" was determined by culturing lymphocytes obtained after separation procedures with recombinant IL-2 (Cetus, Emeryville, CA) at 1000 units/ml for 4 days as described below for the *in vitro* studies. Cytotoxicity was then determined *versus* NK-resistant Raji cells. The target cells were added at 10,000/100 μl culture medium in round-bottomed plates. Effector lymphocytes were added to achieve effector:target ratios of 40:1, 20:1, and 10:1. Assays were performed in triplicate. The percentage of specific cytotoxicity was calculated as the percentage of ^{51}Cr released in the experimental group minus the percentage released in the control (culture medium alone) group. Data are expressed as lytic units, the number of tumor target cells lysed × 100 by 8000 effector cells.

Lymphocyte Subset Analysis. Lymphocyte subset analysis was performed using MAB and fluorescence-activated cell sorter analysis. MAB *versus* pan-T-cell (Leu-1, CD5), T-helper, (Leu-3a, CD4), T-suppressor (Leu-2a, CD8), NK cell (Leu 19, CD56), and B-cell (HLA-DR) determinants were purchased from Becton Dickinson, Mountain View, CA. Analysis using fluorescein-conjugated goat anti-mouse immunoglobulin was performed using an OrthoSystem 50H cytofluorograph according to the recommendations of Becton-Dickinson.

***In Vitro* Studies.** PBMC and PBL separated as described above from healthy volunteers and were cultured in media consisting of RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 1 mM L-glutamine, 1% penicillin-streptomycin solution (GIBCO, Grand Island, NY), 20 nM 4-(2-hydroethyl)-1-piperazineethanesulfonic acid (GIBCO), and 10% pooled heat-inactivated human sera. A range of concentrations of FAA, a range of concentrations of IL-2, as well as FAA plus IL-2 were added to the PBMC and PBL cultures. PBL were cultured at a density of 10^6 cells/ml, and PBMC were cultured at a higher density, 10^7 cells/ml for up to 5 days. LAK cytotoxicity was determined as described above.

Statistical Analysis. The Wilcoxon signed rank matched pairs nonparametric test was used to evaluate the differences between cytolytic activity and lymphocyte subsets at baseline and at various time points after FAA infusion and between cytolytic activity between first and second FAA infusions (8).

RESULTS

Cytotoxicity *in Vivo*. NK-, LAK-, and IL-2-augmented NK activities of cancer patients were evaluated 7 and 1 day prior to FAA infusion to determine baseline activities and at 6, 24, 48, 72, and 96 h after completion of the 3-h FAA infusion. Although there was a good deal of variability in cytolytic activity among patients, baseline determinations for each patient were not significantly different. NK activity of the patients studied was suppressed 24 h after infusion by approximately 50% ($P = 0.01$) (Fig. 1). Suppression was observed at both dose levels. Only in one individual patient (patient 4) was there a modest

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[1] The abbreviations used are: FAA, flavone acetic acid; NK, natural killer; LAK, lymphokine-activated killer; IL-2, interleukin 2; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes, MAB, monoclonal antibody.

Table 1 *Patient characteristics*

Patient	Age (yr)/sex	Cancer	Prior therapy	FAA dose (g/m^2)
1	70/F	Colon	Nafidimide	8.5
2	63/M	Colon	5-FUra,[a] folinic acid	8.5
3	31/F	Rectum	5-FUra, mitomycin, methotrexate	8.5
4	51/M	Colon	5-FUra, cisplatin	10
5	66/M	Head and neck	Cisplatin, 5-FUra, methotrexate, radiation therapy	10
6	59/F	Melanoma	Pentostatin	10
7	70/F	Colon	5-FUra, cisplatin	10
8	67/M	Pancreas	None	10

[a] 5-FUra, fluorouracil.

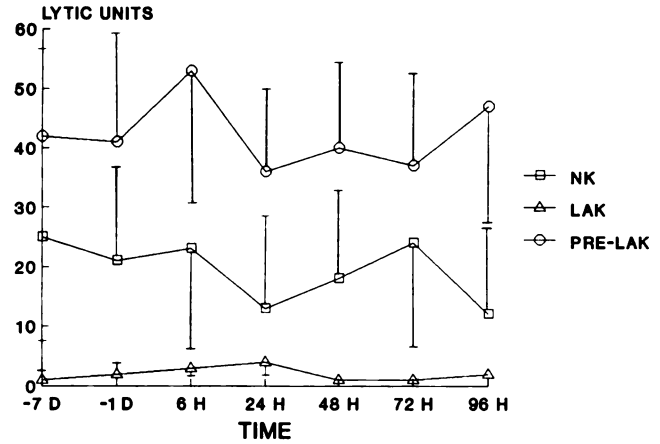

Fig. 1. Effect of FAA *in vivo* on peripheral blood NK-, LAK-, and IL-2-augmented NK activity. Data represent the mean ± SD (*bars*) for the 8 patients studied receiving FAA by 3-h infusion at either 8.5 or 10 g/m^2. Activities were determined 7 and 1 days (*D*) prior to the infusion and at time points (h) after completion of the infusion. Only the decrease in NK activity at 24 h was statistically significant ($P = 0.01$, Wilcoxon signed rank matched pairs nonparametric test).

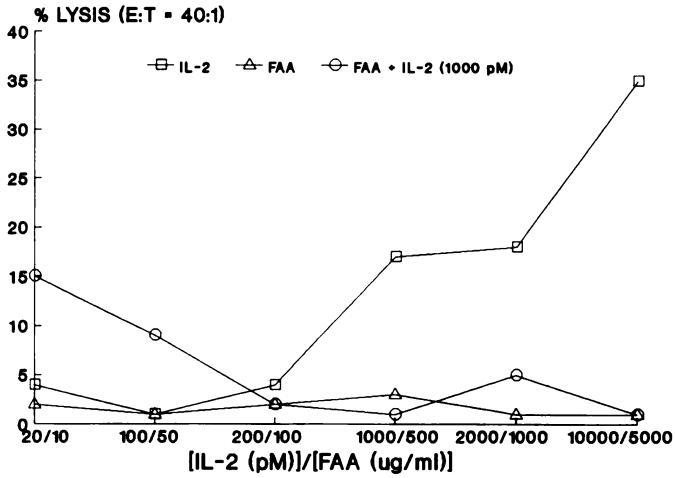

Fig. 2. Effect of FAA, alone and incombination with IL-2, on the generation of LAK activity *in vitro*. PBL collected from 3 healthy volunteers were cultured for 5 days. LAK activity (mean value) is expressed as percentage of lysis at an effector:target cell (*E:T*) ratio of 40:1.

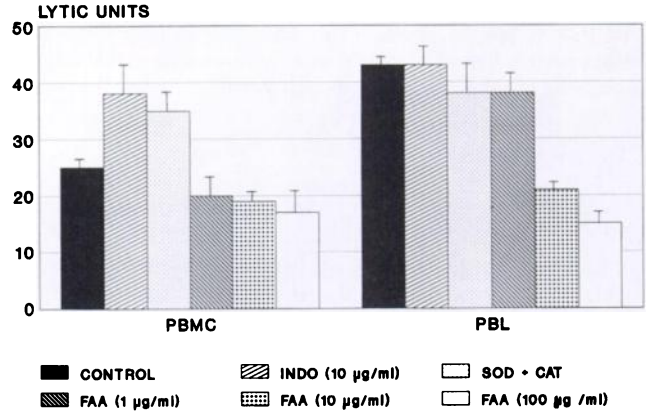

Fig. 3. Effect of FAA, indomethacin (*INDO*), and superoxide dismutase (*SOD*) plus catalase (*CAT*) on the generation of LAK activity from PBMC cultured at 10^7 cells/ml with IL-2 (1000 units/ml). Data represent the mean ± SD (*bars*) of results obtained from cells collected from 3 healthy volunteers.

increase in NK activity (10 lytic units baseline to 16 lytic units at 48 h) after the initial infusion. Induction of LAK activity was not observed in any patient. Statistically significant changes in IL-2-augmented NK activity were also not noted. NK cytotoxicity was evaluated in 6 patients (patients 1–4, 7, and 8) after a second infusion of FAA which was administered 21 to 28 days after the initial infusion. NK activity decreased at 24 h with the second infusion ($P = 0.001$).

Lymphocyte Subsets *in Vivo*. Cell surface marker analysis was performed using a panel of MAB and fluorescence-activated cell sorter of PBL from patients before and after FAA infusions. Total lymphocytes decreased 24 h after infusion. The percentage of NK cells identified with Leu-19 decreased to 65% of baseline ($P = 0.04$) at 24 h. Significant changes in T-cells (CD5), T-helper (CD4), T-suppressor (CD8), and B-cells (HLA-DR) numbers were not effected.

Cytotoxicity *In Vitro*. FAA failed to enhance LAK activity from PBL collected from healthy volunteers after 24 h (data not shown) or after 5 days of culture (Fig. 2) at any concentration. At concentrations ≥100 µg/ml FAA inhibited IL-2-induced LAK activity from PBL (Fig. 2). It has been hypothesized that FAA may enhance lymphocyte cytotoxicity by inhibiting prostaglandin synthesis (5). Flavonoids also have the capacity to enhance lymphocyte cytotoxicity by scavenging reactive oxygen species. We investigated the effects of FAA on LAK generation from monocyte-containing PBMC preparations cultured at a higher density, 10^7 cells/ml. Monocytes are known to suppress LAK generation at densities $>10^6$ cells/ml; prostaglandins and reactive oxygen species have been implicated in this suppression (7). Whereas inhibitors of the metabolism of prostaglandins and reactive oxygen species, indomethacin (10 µg/ml) and superoxide dismutase (10 µg/ml) plus catalase (40 µg/ml), enhanced LAK generation from PBMC cultured at 10^7/ml, FAA had no effects (Fig. 3).

DISCUSSION

In contrast to previously reported studies in mice and in cancer patients, our data suggest that in humans FAA does not enhance NK or LAK activity. The decreases in NK activity and in the number of NK cells identified by Leu-19 MAB observed in the *in vivo* studies and the antagonism observed in the *in vitro* studies suggest that FAA may negatively affect immunological responses in humans. It should be noted that decreased NK activity has also been reported previously in cancer patients receiving FAA (5). Even in studies reporting enhancement of NK activity in individual patients, a majority of patients did not demonstrate enhancement, and consistent significant changes for the group were not noted (5, 6). Previous reports were limited by a single evaluation of baseline activity.

Alterations in FAA administration or in patient selection may be required to demonstrate enhancement of immunological responses. In mice, higher doses appear to be less effective than

lower doses (9). Preclinical studies in mice have also identified a therapeutic window of drug concentrations of 100–600 μg/ml (10). Other investigators have reported enhancement of NK cell activity in individual patients treated using a somewhat lower dose (6.4 g/m^2) than used in this study (6). Although there are interspecies differences in FAA metabolism, the 8.5- and 10-g/m^2 doses we studied are within the order of magnitude of the 0.5 g/kg shown to effectively enhance lymphocyte cytotoxicity in mice. At 24 h after infusion, FAA levels in the patients studied ranged from 3 to 24 μg/ml (peak concentrations of 157 to 410 μg/ml were achieved at 6 h).[2] That almost all of the patients we studied had metastatic cancers refractory to multiple prior combination chemotherapy programs may have also played a role in the lack of immunological response observed. Patients in whom there was enhancement of NK activity in a previous report either had not received chemotherapy or had only received single agent chemotherapy with fluorouracil (5).

The mechanism by which FAA enhances lymphocyte cytotoxicity in mice is unknown. Natural flavonoids can affect cells by various biochemical processes including inhibition of prostaglandin synthesis and scavenging reactive oxygen species (11). Prostaglandins and reactive oxygen species have been implicated in the suppression of lymphocyte cytotoxicity (12, 13). We were able to show enhancement of LAK generation from PBMC cultured at high density using indomethacin and with superoxide dismutase plus catalase. Enhancement using FAA under similar conditions was not observed.

Other immunological mechanisms, such as enhancement of macrophage cytotoxicity or induction of interferon, may be important in the antitumor effects of FAA (14, 15). Enhancement of NK and LAK activity is seen in mice after FAA is administered bicompartmentally, i.v. and i.p. Whether i.p. administration is critical to the immunological effects observed is not known. Although it is generally accepted that the effects of FAA on tumors in mice are indirect, several investigations have suggested nonimmunological mechanisms. Tumor site has been shown to be relevant with regard to sensitivity to FAA. Studies in transplantable murine tumors demonstrated an unusual spectrum of activity against solid tumors but little activity against leukemias (1, 2). Recent reports suggest that FAA induces tumor necrosis by "shutdown" of tumor blood vessels (16). Antitumor effects in mice may be related to FAA-induced alterations in platelet function (17).

Phase II testing of FAA is currently being completed. Thus far, response rates have been disappointing. There is evidence that the immunological effects of FAA in mice are not generalized. Whereas significant enhancement of IL-2 antitumor activity can be demonstrated in BALB/c mice bearing Renca renal adenocarcinoma, the addition of FAA to IL-2 failed to enhance IL-2 antitumor activity in C3HeB/FeJ mice bearing C3H95 adenocarcinoma (18). Interspecies variations in the metabolism of FAA, which may play a central role in FAA activity, is currently being investigated to explain differences observed in response rates observed between humans and mice (19).

[2] M. R. Grever, L. Malspeis, S. P. Balcerzak, *et al.* Phase I investigation of flavone acetic acid, manuscript in preparation.

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