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FLAVONE-8-ACETIC ACID AUGMENTS SYSTEMIC NATURAL KILLER CELL ACTIVITY AND SYNERGIZES WITH IL-2 FOR TREATMENT OF MURINE RENAL CANCER[1]

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The investigational drug flavone-8-acetic acid (FAA) potently augments NK activity in the spleen, liver, lungs, and peritoneum in a dose-dependent manner after i.v. or i.p. administration. Augmented NK activity peaks by 24 h after FAA injection and returns to normal after 6 days. Combined treatment of established murine renal cancer with FAA and rIL-2 results in up to 80% long term survival whereas FAA or rIL-2 alone were unable to induce any long term survivors. The optimal dose of rIL-2 required for use with FAA was in the range of 10,000 to 30,000 U/day. Further studies demonstrated that the regimen of FAA plus rIL-2 administration that was effective in treating established murine renal cancer also induced a more potent augmentation of NK activity than did either FAA or rIL-2 alone. Subsequent studies revealed that the therapeutic effectiveness of FAA plus rIL-2 was significantly reduced when tumor-bearing mice were treated with anti-asialo GM_1 serum. These results are consistent with a role for augmented NK activity in the therapeutic effects of FAA plus rIL-2 murine renal cancer. In addition, these studies demonstrate that FAA and rIL-2 is a useful approach for cancer treatment in that subtoxic doses of rIL-2 can be used and significant anti-tumor efficacy occurs even without accompanying adoptive immunotherapy.

Recent studies have demonstrated that the adoptive transfer of specifically immune or broadly cytotoxic lymphocytes generated in the presence of human rIL-2 can result in the regression of established tumors in mice and humans (1–5). Similarly, the administration of rIL-2 alone, in the absence of AIT,[3] also produced some anti-tumor effects in mice (3) and humans (6). However, the use of AIT and rIL-2 to treat cancer patients is a complicated, expensive, and toxic form of therapy. This realization has stimulated interest in using rIL-2 in combination with other BRM or with chemotherapeutic drugs in the hope that more convenient and less toxic cancer treatment approaches can be developed.

The investigational drug FAA was initially selected for clinical testing in cancer patients based upon a) an unusual spectrum of activity against murine solid tumors refractory to conventional chemotherapeutic agents (7–9) and b) favorable preclinical pharmacologic and toxicologic profiles (7, 8). Because this agent is somewhat more effective in vivo than in vitro (7, 8), we speculated that FAA might act as a BRM, as well as a direct chemotherapeutic agent. The results presented in this report demonstrate that the administration of FAA to mice results in a potent systemic augmentation of NK activity. The results also show that FAA plus rIL-2 further augments NK activity and exhibits dramatically improved therapeutic effects, comparable to those achieved previously with chemotherapeutic drugs and AIT (5), against a murine renal cancer.

MATERIALS AND METHODS

Mice. Male BALB/C mice were housed under specific pathogen-free conditions and were used routinely at 7 to 10 wk of age.

BRM. FAA was synthesized by Lyonnaise Industrielle Pharmaceutique (LIPHA, Lyon, France) and acquired through the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Frederick, MD). The rIL-2 (3×10^6 Biological Response Modifiers Program units/mg protein) was generously supplied by Cetus Corporation (Emeryville, CA). Polyinosinic-polycytidylic acid and poly-L-lysine stabilized in carboxymethyl cellulose was generously provided by Dr. Hilton Levy, National Institute of Allergy and Infectious Diseases (Frederick, MD). Anti-asGM$_1$ serum was purchased from the Wako Pure Chemical Co. (Dallas, TX). This antiserum has been previously shown to inhibit NK activity after in vivo administration (10–12). All reagents were diluted in HBSS for administration to mice.

Assessment of NK activity. Augmentation of NK activity by FAA and/or rIL-2 was assessed in a 4-hr ^{51}Cr release assay against the YAC-1 tumor target. Effector leukocytes were isolated from spleen, peritoneal cavity, lungs, and liver as previously described (13, 14). NK activity was expressed as either

$$\% \text{ specific cytotoxicity} = \frac{\text{cpm released from test well} - \text{cpm released from target cells alone}}{\text{total SDS-solubilized cpm}} \times 100$$

or LU/1×10^7 cells, with one LU being the number of effector cells required to lyse 20% or 40% of the target cells. Statistical compari-

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[3]Abbreviations used in this paper: AIT, adoptive immunotherapy; BRM, biologic response modifiers; FAA, flavone-8-acetic acid; anti-asGM$_1$, anti-asialo GM$_1$.

sons in NK activity were performed by student's *t*-test.

Tumor model. The model used for these studies is the Renca renal adenocarcinoma, a tumor that originated spontaneously and is maintained by serial transplant in BALB/C mice (15). The growth characteristics of this tumor have been previously described in detail (5). After injection of 1×10^5 tumor cells under the renal capsule, the solid tumor mass develops rapidly with direct extension to the peritoneal cavity by days 7 to 9 and metastasis to regional lymph nodes and liver shortly thereafter. Surgical resection of the primary tumor-bearing kidney is potentially curative before day 8 but not thereafter when mice succumb to peritoneal carcinomatosis and subsequent metastatic disease. FAA was administered by injection of 125 mg/kg i.v. and 125 mg/kg i.p., whereas various doses of rIL-2 were delivered by the i.p. route. Routinely, FAA was administered 2 to 4 h after nephrectomy of the primary tumor-bearing kidney on day 11, and rIL-2 was administered once per day for 4 successive days beginning on the day after nephrectomy and FAA treatment. Statistical analysis of survival data was performed by χ^2 test.

RESULTS

Augmentation of NK activity after administration of FAA. Table I illustrates that a single i.v. dose of FAA (250 mg/kg), which has been shown previously to be both active against murine solid tumors and relatively nontoxic (7–9), strikingly augmented NK activity in the spleen and liver ($p < 0.01$). The maximum enhancement (51.2 and 236 LU, respectively) of NK activity occurred in both tissues by day 1, remained significantly ($p < 0.01$) augmented through day 3, and returned to near background levels by day 6. Further studies shown in Table II illustrate that NK stimulation ($p < 0.01$ to 0.001) by FAA was similarly demonstrable in the lungs and peritoneum and that the effects were dose dependent in all tissues examined. Interestingly, levels of NK activity induced by the highest tested dose (250 mg/kg) of FAA were comparable to those induced by the positive control, polyinosinic-polycytidylic acid and poly-L-lysine stabilized in carboxymethyl cellulose, a very effective BRM in augmenting systemic NK activity.

TABLE I

Augmentation of NK activity in the spleen and liver after administration of FAA

Treatment In Vivo[a]	% Cytotoxicity[b]	
	Spleen	Liver
None	12 ± 1 (10.3)	9 ± 3 (7)
FAA (−1 day)	38 ± 2 (51.2)[c]	66 ± 4 (236.0)[c]
FAA (−3 days)	25 ± 3 (34.5)[d]	53 ± 2 (150.8)[c]
FAA (−6 days)	16 ± 4 (17.4)	18 ± 3 (21.3)[d]

[a] FAA (250 mg/kg) was administered i.v. 1, 3, or 6 days before the assessment of NK activity in leukocytes isolated from the spleen and liver. All groups consisted of five mice and NK activity was assessed against YAC-1 at E/T ratios of 30:1, 15:1, and 7:1. Data is presented as the mean ± SD for an E/T of 30:1.

[b] Numbers in parentheses, $LU_{20}/1 \times 10^7$ leukocytes.

[c] Significantly greater than control, $p < 0.001$.

[d] Significantly greater than control, $p < 0.01$.

Treatment of murine renal cancer by FAA and/or rIL-2. Because 250 mg/kg FAA markedly enhanced NK activity both in lymphoid and nonlymphoid sites (Tables I and II) and NK cells constitute a major precursor for lymphokine-activated killer activity (16–19) generated by rIL-2, we performed experiments to determine whether FAA and rIL-2 could have additive or synergistic anti-tumor activity when used in combination. Figure 1*A* shows that a single bicompartmental administration of FAA alone (125 mg/kg i.v. plus 125 mg/kg i.p.) or multiple daily i.p. administrations of either 10,000 U or 100,000 U of rIL-2 alone only slightly prolonged the survival of Renca-bearing mice. In contrast, the combination of FAA and 10,000 U rIL-2 rendered 80% of the tumor-bearing mice free of grossly visible disease through 120 days. Moreover, when FAA-treated mice were tested with a range of daily doses of rIL-2, long term survivors were obtained with as little as 100 to 1000 U of rIL-2 per day (5 of 19 survivors) Figure 1*B*). The survival curve is statistically significantly improved over the control curve at doses of rIL2 ≥1000 U/mouse. Significantly improved long term survival was observed for 1000 U ($p < 0.05$), 10,000 U ($p < 0.05$), 30,000 U ($p < 0.005$), and 100,000 U ($p < 0.005$). Overall, these results demonstrate that the use of FAA in association with moderate doses of rIL-2 affords appreciably improved long term survival of mice bearing murine renal cancer as compared with treatment with either FAA or rIL-2 alone.

Augmentation of NK activity by FAA plus rIL-2. Because FAA has been demonstrated to augment NK activity (Tables I and II) and rIL-2 has also been demonstrated to augment NK activity, we performed studies to determine whether the combination of FAA plus rIL-2 would augment NK activity to a greater extent than would FAA or rIL-2 alone. The results shown in Table III demonstrate that FAA plus rIL-2 induce significantly ($p < 0.01$) higher levels of NK activity (150 LU) than either FAA (40 LU) or rIL-2 (18 LU) alone in blood. Similarly, splenic NK activity was significantly ($p < 0.01$) increased from 106 LU after FAA alone and 110 LU after rIL-2 alone to 938 LU after administration of FAA plus rIL-2. Hepatic NK activity was increased almost 20-fold to 1744 LU for FAA plus rIL-2 from 91 LU and 92 LU for FAA or rIL-2 alone, respectively. These results demonstrate that FAA plus rIL-2 more potently augmented NK activity than did FAA or rIL-2 alone and are consistent with the hypothesis that this augmentation of NK activity may contribute to the therapeutic effects of the FAA plus rIL-2 combination.

Inhibition of anti-tumor effectiveness of FAA plus rIL-2 by pretreatment with anti-asGM$_1$ serum. Because

TABLE II

Augmentation of NK activity in different sites after administration of various doses of FAA

Treatment In Vivo (Dose)[a]	% Cytotoxicity[b]			
	Spleen	Peritoneum	Liver	Lungs
HBSS	10 ± 2 (6.4)	6 ± 2 (2.1)	14 ± 2 (7.2)	8 ± 1 (4.0)
Poly ICLC (0.5 mg/kg)[c]	71 ± 6 (64.2)[d]	57 ± 4 (137.4)[d]	60 ± 5 (211.7)[d]	38 ± 4 (84.3)[d]
FAA (250 mg/kg)	53 ± 1 (56.4)[d]	51 ± 3 (108.2)[d]	58 ± 2 (103.4)[d]	34 ± 2 (39.9)[d]
FAA (125 mg/kg)	49 ± 4 (37.6)[e]	42 ± 3 (94.8)[d]	22 ± 3 (20.1)[e]	12 ± 1 (10.2)
FAA (25 mg/kg)	11 ± 1 (7.3)	30 ± 1 (76.1)[d]	6 ± 3 (5.5)	ND

[a] BRM were administered i.v. (for assessment of NK activity in spleen, liver, and lungs) or i.p. (for assessment of NK in the peritoneum) 24 h before the isolation of effector leukocytes. All groups consisted of five mice and NK activity was assessed against YAC-1. Data is presented as mean ± SD.

[b] Numbers in parentheses, $LU_{20}/1 \times 10^7$ leukocytes.

[c] Poly ICLC, polyinosinic-polycytidylic acid and poly-L-lysine stabilized in carboxymethyl cellulose.

[d] Significantly greater than HBSS control, $p < 0.001$.

[e] Significantly greater than HBSS control, $p < 0.01$.

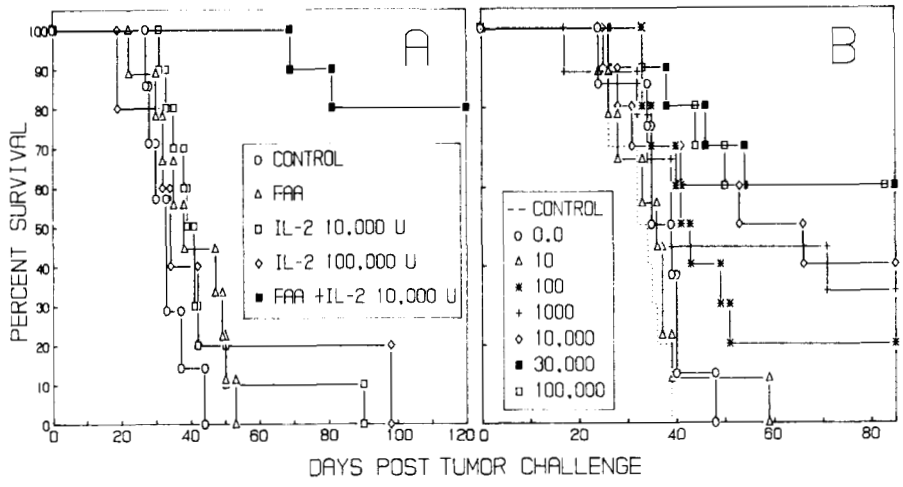

Figure 1. Effect of treatment with FAA and/or rIL-2 on survival of Renca-bearing mice. BALB/C mice (8 to 10/group) were injected i.v. with 1×10^5 Renca tumor cells on day 0. On day 11 the tumor-bearing kidney was removed and 2 to 4 h later FAA (125 mg/kg i.v. + 125 mg/kg i.p.) was administered to appropriate groups. Subsequently, beginning on day 12, some of the FAA-pretreated mice and two groups of previously untreated mice received four daily i.p. injections of rIL-2 at doses of either 10,000 or 100,000 U/day (*A*). Alternatively, FAA-treated mice received increasing doses of rIL-2 ranging from 10 to 100,000 U (B). The control group in *B*, represented by the *dotted line*, received no FAA or rIL-2. Mice were then monitored for survival and statistical analyses performed by χ^2 test.

TABLE III

Augmentation of NK activity by in vivo administration of FAA and/or rIL-2

Expt.	Tissue	Treatment[a]	LU[b]
1	Blood	HBSS	3
		FAA	40
		rIL-2	18
		FAA + rIL-2	150[c]
2	Spleen	HBSS	15
		FAA	106
		rIL2	110
		FAA + rIL-2	938[c]
	Liver	HBSS	11
		FAA	91
		rIL-2	92
		FAA + rIL-2	1744[c]

[a] BALB mice were injected i.v. with FAA (250 mg/kg) and/or i.p. with three daily doses of 30,000 U/day rIL-2, beginning 24 h after the administration of FAA. Blood, spleens, and livers were harvested 24 h after the last injection of rIL-2, and leukocytes were isolated for determination of NK activity.

[b] Data are expressed at LU_{20} for Experiment 1 and as LU_{40} for Experiment 2.

[c] Significantly greater than FAA alone or rIL-2 alone, $p < 0.01$.

TABLE IV

Effect of anti-asGM$_1$ serum pretreatment on anti-tumor efficacy of FAA + rIL-2

Treatment[a]	Survivors/Total	% Survival
HBSS	0/10	0
FAA + rIL-2	8/14	57
Anti-asGM$_1$ serum + FAA + rIL-2	2/13[b]	15

[a] BALB/C mice were nephrectomized 11 days after intrarenal injection of 1×10^5 Renca cells. FAA (200 mg/kg) was administered i.v. 4 h after nephrectomy and rIL-2 (30,000 U/day) was administered i.p. for 3 days beginning 24 h after FAA administration. Anti-asGM$_1$ serum (0.2 ml of a 1/10 dilution) was administered i.v. 2 h before FAA, 24 h after FAA, and 72 h after FAA.

[b] Significantly less than FAA + IL2 positive control, $p < 0.025$.

anti-asGM$_1$ serum has been widely demonstrated to inhibit NK activity with some degree of selectivity (10–12, 14, 18, 20) we performed studies to determine whether administration of this antiserum, before and during FAA plus rIL-2 treatment, could inhibit the therapeutic effects of FAA plus rIL-2 for murine renal cancer.

The results shown in Table IV confirm the data in Figure 1 showing that the FAA plus rIL-2 combination is effective in treating established murine renal cancer inasmuch as 8 of 14 (57%) mice were rendered disease-free by this regime. However, when tumor-bearing mice were also treated with several doses of anti-asGM$_1$ serum, the therapeutic effect of FAA was significantly ($p < 0.025$) reduced to 15% (2 of 13 survivors) when compared with the FAA plus rIL-2 positive control group. These results demonstrate that asGM$_1^+$ cells contribute to the therapeutic effects of FAA plus rIL-2 against murine renal cancer.

DISCUSSION

Recent studies have indicated that rIL-2 has notable anti-tumor activity when used alone in tumor-bearing mice (1–3, 5) and in certain human cancer patients (4, 6). However, the therapeutic efficacy of rIL-2 appears to be enhanced when used in conjunction with AIT (3–5). In humans, the administration of large amounts of rIL-2 induces a variety of severe and dose-limiting toxic side effects (21). Therefore, much attention has recently focused on alternative strategies that could exploit the therapeutic benefits of rIL-2 while decreasing the expense and logistic difficulties associated with AIT, as well as decreasing the toxic sequelae associated with high dose rIL-2 therapy. We have previously noted that the Renca murine renal cancer could be successfully treated by a therapeutic regimen that combined doxorubicin and AIT with rIL-2 (5, 22). This approach offered the advantage of requiring daily administration of a moderate amount of rIL-2 instead of the larger amounts required for therapeutic effects with rIL-2 alone. However, the identification of agents, either cytokines, noncytokine BRM, or chemotherapeutic drugs, that could successfully act in concert with moderate amounts of rIL-2 in the absence of AIT would be quite useful.

The investigational agent FAA has been shown to have direct anti-tumor activity against a broad spectrum of solid tumors in mice (7–9). The mechanism for this anti-tumor effect has not been defined. The present report demonstrates that FAA is able to augment NK activity systemically (spleen, liver, lungs, and peritoneum), extending a recent observation that FAA augmented splenic NK activity (23). The finding of FAA-augmented NK activity in tissue sites such as the lungs and liver may be of relevance to the treatment of metastases in these organs because NK cells can mediate anti-metastatic effects in those sites (20, 24). This observation of systemic NK augmentation by FAA, coupled with recent evidence that lymphokine-activated killer activity can be generated by treatment of NK cells with rIL-2 (16–19), caused us to investigate whether FAA and rIL-2 might have

additive or synergistic anti-tumor activity. The results shown in Figure 1 clearly demonstrate a synergistic anti-tumor effect of these two agents for murine renal cancer and suggest that FAA in association with moderate doses of rIL-2 may be a more useful approach to the treatment of cancer than administration of high doses of rIL-2 alone. Recent studies have also shown that FAA and rIL-2 is also effective in eradicating established experimental hepatic metastases and that the inclusion of AIT in the treatment regimen does not further increase survival (R. L. Hornung and R. H. Wiltrout, manuscript in preparation).

The mechanism by which FAA and rIL-2 complement each other for the treatment of murine renal cancer is under current investigation. It is clear that FAA plus rIL-2 augments NK activity to a greater extent than does FAA or rIL-2 alone (Table III). Thus this increase in NK augmentation correlates with the enhanced anti-tumor activity observed after treatment with FAA plus rIL-2. There are several ways in which the increased activity of NK cells induced by FAA plus rIL-2 could translate into enhanced anti-tumor effects. First, one could envision that NK cells might participate by direct cytolysis of tumor. Second, NK cells have been shown to produce and secrete a variety of potent immunoregulatory molecules (25, 26) that could synergize with IFN, rIL-2, or rIL-2-induced cytokines, for induction of anti-tumor effects. There is preliminary evidence that FAA induces production of several cytokines (R. L. Hornung, H. A. Young, W. J. Urba, and R. H. Wiltrout, manuscript in preparation) (27), and rIL-2 has been shown to induce cytokines as well (26, 28). It is also possible that FAA alters the subset composition in various organs, such that the percentage of NK-active large granular lymphocytes is increased, which would lead to the increase in NK activity. Studies to investigate the ability of FAA to alter subset composition are in progress. Irrespective of the actual mechanism by which NK cells could contribute to the therapeutic effect of FAA plus rIL-2 there is evidence that they do contribute, because the administration of anti-asGM$_1$ serum does abrogate the therapeutic effect of FAA plus rIL-2. The augmentation of NK activity by FAA is an indirect effect because this agent does not boost NK activity in mice or humans in vitro (R. H. Wiltrout and J. R. Ortaldo, manuscript in preparation). Thus, it seems likely that the induction of NK activity, and perhaps therapeutic effects, is mediated by metabolites of FAA or by cytokines induced by FAA. Studies using analogues of FAA with varying therapeutic and immunomodulating activity are underway to address these issues. Additional possible mechanisms for the anti-tumor effectiveness of FAA and rIL-2 include removal of suppressor cells (1, 2) or induction of increased immunogenicity.

Overall, these studies demonstrate that FAA potently augments NK activity in all tissue compartments sampled. Furthermore, FAA and rIL-2 mediate synergistic anti-tumor effects when used together against murine renal cancer. Because preclinical models have usefully predicted clinical utility with other treatment approaches using rIL-2, these results suggest that FAA and rIL-2 should be expeditiously evaluated for the treatment of cancer in humans.

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