AUGMENTATION OF NATURAL KILLER ACTIVITY, INDUCTION OF IFN AND DEVELOPMENT TUMOR IMMUNITY DURING THE SUCCESSFUL TREATMENT OF ESTABLISHED MURINE RENAL CANCER USING FLAVONE ACETIC ACID AND IL-2

RONALD L. HORNUNG, TIMOTHY C. BACK,* DANIEL S. ZAHARKO,† WALTER J. URBA,* DAN L. LONGO,* AND ROBERT H. WILTROUT3

From the Laboratory of Experimental Immunology, †Biological Response Modifiers Program, and ‡Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD 21701; and *Clinical Immunology Services, Program Resources, Inc., National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD 21701

The investigational drug flavone acetic acid (FAA) has been previously shown to systemically augment NK activity in vivo in normal mice within 24 h of i.p. or i.v. administration. The current study investigates the ability of FAA, and/or rIL-2, to augment NK activity and antitumor responses in mice bearing murine renal cancer (Renca). The results demonstrate that FAA potently augments NK activity in the blood, spleen, and liver of Renca-bearing mice and that the administration of rIL-2 in addition to FAA results in a further augmentation of NK activity over that observed with FAA alone. Renca-bearing mice treated with FAA (200 to 250 mg/kg) plus rIL-2 exhibited a significantly increased incidence of long term survivors (59%) over that observed following treatment with FAA (0%) or rIL-2 (5%) alone. Therapeutic synergy between FAA and rIL-2 was observed against primary tumors, minimal residual disease, and experimental-induced pulmonary metastases. Mice cured of Renca by FAA plus rIL-2 treatment were largely resistant to rechallenge with Renca suggesting a role for T lymphocytes. The augmentation of NK activity and the therapeutic effects of FAA coincided with the rapid induction of high titers of serum IFN of the α/β type within 4 h of FAA administration. Subsequent studies demonstrated that the contribution of FAA could be partially replaced by the administration of several doses of human rIFN-α A/D Bgl before the initiation of rIL-2 administration. The observed synergistic antitumor effects of FAA plus rIL-2 coincided with the augmentation of NK activity, induction of IFN-α/β, and induction of long lasting tumor immunity. Overall, these results suggest that this approach may obviate the need for adoptive immunotherapy in association with rIL-2 administration for at least some tumor types.

The availability of large amounts of purified human rIL-2 generated by molecular cloning (1), has permitted studies demonstrating that rIL-2 has pre-clinical and clinical anti-tumor activity when used as a single agent (2–4), or when administered in conjunction with the adoptive transfer of specifically immune cytotoxic cells (5, 6) or broadly cytotoxic LAK cells generated by in vitro coculture with rIL-2 (7–9). The therapeutic efficacy of rIL-2 is maximal when it is administered in conjunction with adoptive transfer of IL-2-stimulated effector cells (7, 10, 11). Unfortunately, this type of AIT is very expensive and associated with significant toxicity (4, 8, 12) at the high doses of rIL-2 which may be required for therapeutic activity. Consequently, there is considerable interest in developing combination approaches to cancer treatment which could use less toxic regimes of rIL-2. Previous studies from our laboratory have used such a combination approach to demonstrate that the effectiveness of AIT was synergistically enhanced when it was used in combination with the chemotherapeutic drug, doxorubicin hydrochloride (Dox) for treatment of murine renal cancer (9, 13).

Because of the expense, complexity, and/or toxicity associated with current AIT protocols we initiated studies to determine whether chemotherapeutic drugs or BRMs in combination with rIL-2 could be successful in the absence of AIT. The investigational drug FAA was chosen for study in combination with rIL-2 on the basis of previous observations that it has single agent antitumor activity against slow growing solid tumors in mice (14–16), and that it also has the ability to systemically augment NK activity in normal mice (17, 18). Therefore, FAA appears to function as a chemotherapeutic drug and also as a BRM, and might thus synergize with AIT or rIL-2 because it could function as a debulking agent, as well

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Address correspondence and reprint requests to Dr. Robert H. Wiltzut, Laboratory of Experimental Immunology, NCI-FCRF, Bldg. 560, Room 31-93, Frederick, MD 21701-1013.

Abbreviations used in this paper: LAK, lymphokine-activated killer; FAA, flavone acetic acid; cpm, counts/mln; asGM, anti-asialo GM; BRM, define please; AIT, adoptive immunotherapy; NK, natural killer cell.
as a potentiator of the activity of NK cells (17, 18) which have been shown to contribute substantially to the generation of LAK activity by rIL-2 (19–22). Preliminary studies have confirmed that FAA and rIL-2 could synergize for the treatment of murine renal cancer (17).

In the present report we have demonstrated that FAA potently augments NK activity in tumor-bearing mice, and that the FAA + rIL-2 combination is effective against both the primary Renca tumor in the absence of surgery, and established experimental lung metastases. Additional experiments have investigated the activities of NK cells, specific immunity, and cytokine induction in the therapeutic activity of the FAA/rIL-2 combination.

### MATERIALS AND METHODS

**Animals.** Male euthymic and athymic BALB/c mice were obtained from the Animal Production Area, NCI-Frederick Cancer Research Facility (Frederick, MD), housed in a specific-pathogen free animal facility, and routinely used at 7 to 10 wk. of age.

**Tumor cell lines.** The Renca renal adenocarcinoma of spontaneous origin (23, 24) was kindly provided by Drs. E. J. Pontes and G. F. Murphy (Roswell Park Memorial Institute, Buffalo, NY) and maintained in BALB/c mice by serial i.p. passage. YAC-1, a Moloney murine virus-induced lymphoma of A/Sn origin (25) was used for assessment of NK activity, and P815 was used to assess LAK activity (22). All tumors were maintained in continuous cell culture in complete RPMI 1640 medium (Meditech, Washington, DC) supplemented with 10% FBS (Sterile systems, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), and l-glutamine (20 mM).

All tumor cell lines were shown to be free of mycoplasma (Flow Laboratories, MacLean, VA) as well as pneumonia and minute virus of mice, mouse hepatitis and adenovirus, and Sendai, encephalomyocarditis, and lactic dehydrogenase-elevating viruses (Animal Health Diagnostic Laboratory, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD).

**Reagents.** FAA was generously provided by S. Léonard in lyophilized form by Lyonnais Industrielle Pharmaceutique (LIPHA. Lyon, France). Human rIL-2 (3 × 10^6 IU/mg) was generously supplied in lyophilized form by Cetus Corp. (Emeryville, CA). Recombinant human IFN-α A/D Bgl (rIFN-α A/D bgl) was a generous gift from Hoffmann-LaRoche, Inc., Nutley, NJ at a specific activity of 7.9 × 10^6 U/ml. Polyclonal rabbit anti-mouse IFN-α/β and rat anti-mouse IFN-γ globulins were purchased from Lee BioMolecular Research Laboratories, Inc. (San Diego, CA).

**Generation of LAK cells.** Splenocytes from BALB/c mice were aspirationally removed from animals and mechanically dissociated into a cell suspension with a Stomacher Lab Blender 80 (Tekmar, Cincinnati, OH). Debris and cell aggregates were sedimented for 10 min at 1 g. The cell suspension was decanted and centrifuged at 250 × g for 10 min, and the red blood cells lysed with the suspension with distilled H2O. The resuspended cell suspension was passed through sterile gauze and the leukocytes washed twice with HBSS.

The leukocytes were then resuspended to 2 × 10^6 cells/ml and incubated for 5 to 5 days in vitro with 1000 U/ml of rIL-2 in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (20 mM), 1 μM sodium pyruvate (GIBCO Laboratories, Grand Island, NY), 0.1 mM non-essential amino acids (GIBCO), and 2-ME (Sigma, St. Louis, MO).

Cell-mediated cytotoxicity assays.** Serum IFN neutralization with FAA or rIL-2 was assessed as previously described (22, 26) by incubating varying numbers of effector cells in triplicate with 1 × 10^5 51Cr-labeled YAC-1 or P815 tumor targets, respectively, in round bottom, 96-well microtiter plates at 37°C for 4 h in RPMI 1640 medium supplemented with 5% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (20 mM), and 20 mM HEPES buffer. Effector leukocytes were obtained from blood, spleen, or liver and prepared as previously described (26). Supernatants were removed from the wells with a vacuum manifold (Flow Laboratories, Flow Lab. Inc., McLean, VA) and counted on the Beckman Gamma 5500 gamma radiation counter (Beckman Instruments, Inc., Irvine, CA). Percent specific cytotoxicity was calculated by the following formula:

\[
\text{% Specific cytotoxicity} = \left( \frac{\text{cpm released from target cells alone}}{\text{cpm released from test well}} - \frac{\text{cpm released from target cells alone}}{\text{total SDS solubilized cpm}} \right) \times 100
\]

**Experimental model.** Intraperitoneal tumor nodules were excised under sterile conditions from Renca-bearing mice and single cell suspensions prepared by mechanical dissociation and filtration of the resulting tumor cell suspension through gauze. Intrarenal tumor inoculations were maternally performed by injecting 1 × 10^6 tumor cells in 0.1 ml of HBSS (Meditech, Washington, DC) as previously described (24). The tumor grows progressively under the renal capsule and extends to the peritoneal cavity within 7 to 9 days, with metastases formed in the regional lymph nodes and liver shortly thereafter (24).

In survival experiments the primary tumor mass was surgically removed by nephrectomy 11 days after implantation. Within several hours of the nephrectomy mice were given a single bicompartimental (50% of the dose i.v. and 50% of the dose i.p.) administration of FAA. One day after FAA administration mice received the first of 4 daily i.p. injections of various doses of rIL-2. Tumor progression and survival were monitored on a daily basis.

Experimentally induced renca lung metastases were produced by i.v. injection of 2.5 × 10^6 tumor cells in 0.2 ml of HBSS. FAA or the combination of FAA plus rIL-2 therapy was initiated either 3 or 6 days after i.v. tumor injection. Unless noted all life table figures display data from a single representative experiment that has been repeated on at least three separate occasions, yielding similar results.

**IFN assay.** Serum IFN activity was determined using the vesicular stomatitis viral inhibition assay (27), with 1 U of IFN equal to the amount of IFN in 1 ml of sample that reduces viral lysis by 50% in the bioassay (Clinical Immunology Services, Program Resources Inc., NCI-Frederick, Frederick, MD) The bioassay was performed by incubating serum samples for 1 h in a 1/25 or 1/10 dilution of anti-α plus -β or anti-IFN-γ serum, respectively.

**Statistics.** Experimental groups consisted of 8 to 10 mice. All observation times extended to at least 120 days before reporting. Survival statistics were performed using x^2 analysis and survival curve analysis was performed by the Mann-Whitney test.

### RESULTS

**Augmentation of NK activity in various organs of renca-bearing mice.** Previous studies have demonstrated that FAA potently augments NK activity in normal mice (17, 18). The present studies were undertaken to determine whether FAA would induce a similar augmentation of NK activity in tumor-bearing mice, to determine how rapidly such a response was induced, and how long it was maintained. The results shown in Figure 1 demonstrate that FAA increased NK activity in the spleen, blood, and liver in non-nephrectomized mice bearing intra-renal Renca. NK activity is significantly (p < 0.001) enhanced as early as 3 h after injection of FAA in the spleen and liver. Peak activity occurs by 24 h and declines to near background by day 6. Similarly, significant augmentation of NK activity was observed in blood by 6 h after FAA injection (p < 0.001); peak levels were attained by 24 h and augmented activity persisted for 6 days after FAA administration. Further studies shown in Table 1 confirm that both FAA (69 LU) and rIL-2 (51 LU) significantly (p < 0.001) augment NK activity in renca-bearing mice. However, the combination of FAA plus rIL-2 significantly (p < 0.001) augments NK activity three- to fivefold (230 LU) over that obtained with FAA or rIL-2 alone. These results demonstrate that FAA rapidly augments systemic NK activity in renca-bearing mice, and that the combination of FAA + rIL-2 more potently augments NK activity than does either FAA or rIL-2 alone.

**Treatment of established Renca by FAA in combination with rIL-2.** Preliminary studies have demonstrated that FAA and moderate doses of rIL-2 synergized for the treatment of Renca (17). The studies presented in Figure
Figure 1. Time course of the FAA-induced augmentation of NK activity in leukocytes isolated from the liver, spleen, and blood in mice bearing an 11-day intra-renal Renca tumor. NK activity was determined at various time points after a single administration of 200 mg/kg of FAA (split equally i.v. and i.p.).

Table 1
Augmentation of splenic NK activity by FAA and/or rIL-2 in Renca-bearing mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NK Activity (LU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>9</td>
</tr>
<tr>
<td>FAA</td>
<td>69</td>
</tr>
<tr>
<td>rIL-2</td>
<td>81</td>
</tr>
<tr>
<td>FAA plus rIL-2</td>
<td>230</td>
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</table>

*Rena-bearing mice were nephrectomized several hours before administration of FAA and/or rIL-2. These data are from a representative experiment which has been performed with similar results on three separate occasions.

*a Significantly greater than HBSS control, p < 0.001.
*b Significantly greater than FAA or rIL-2 groups, p < 0.001.

Figure 2. Survival of Renca-bearing mice after treatment with FAA and/or rIL-2. BALB/c mice were injected intra-renal with 1 x 10⁵ Renca tumor cells on day 0. On day 11 the tumor-bearing kidney was removed from all mice and 2 to 4 h later FAA (200 to 250 mg/kg split equally i.v. and i.p.) was injected to appropriate groups. Beginning on day 12, some of the FAA pre-treated mice and previously untreated mice received 3 daily i.p. injections of 30,000 U of rIL-2. Another group of FAA pre-treated mice also received three daily injections of LAK cells (3 x 10⁷ cells split equally i.v. and i.p.) concurrently with 4 daily i.p. injections of 30,000 U of rIL-2. Lysite activity against P815 was routinely monitored to assure good LAK activity in the adoptively transferred cell population. The number of mice in the HBSS control, FAA, rIL-2, FAA plus LAK, and FAA plus LAK plus rIL-2 treatment groups were 69, 64, 20, 78, and 37, respectively.

Results convincingly demonstrate that the combination of FAA plus rIL-2 is superior to either agent alone, in the treatment of Renca. Interestingly, the addition of adoptive immunotherapy with LAK cells to the FAA plus rIL-2 treatment did not improve the incidence of long term survivors over that observed with FAA plus rIL-2 alone. Further studies demonstrated that multiple daily injections of rIL-2 at doses as high as 100,000 U/day had no significant effect on survival of Renca-bearing mice (data not shown).

Having observed that the combination of FAA plus rIL-2 appears to be quite effective in nephrectomized Renca-bearing mice bearing minimal residual disease, we performed experiments to determine whether this approach would also work against a larger tumor burden. Pooled data from 2 experiments (Fig. 3), demonstrate that FAA plus rIL-2 significantly improved long term survival to 40% (8/20) of non-nephrectomized and 60% (12/20) of nephrectomized day 11 Renca-bearing mice, p < 0.03 and < 0.001, respectively. Both FAA or rIL-2 alone have been tested in previous experiments, and shown to have no therapeutic efficacy in non-nephrectomized Renca-bearing mice (data not shown). These results demonstrate that a single cycle of the FAA plus rIL-2 combination is effective against the primary tumor in the kidney, as well as against residual disease.
Inasmuch as a frequent impediment to the therapy of human tumors is the presence of metastases, we also performed studies to determine whether the combination of FAA plus rIL-2 had therapeutic activity against experimentally induced lung metastases. In these studies mice with pulmonary metastases, selectively produced by i.v. injection of $2.5 \times 10^5$ Renca cells, were treated beginning either on day 3 or day 6. Preliminary studies had demonstrated that FAA alone was able to prolong survival times slightly, whereas rIL-2 alone had no effect on survival of mice with experimentally induced metastases (data not shown). Therefore, we performed the experiments shown in Figure 4 to determine whether the combination of FAA plus rIL-2 would afford better therapeutic results for established metastases than FAA alone. The results were consistent with the data presented in Figures 2 and 3, in that FAA plus rIL-2 rendered 70% of the mice with day 3 lung metastases disease free. Although none of the mice treated with FAA alone survived, the mean survival time of these mice was significantly ($p < 0.001$) increased from 38.7 days to 56.4 days. Treatment of mice bearing more established day 6 lung metastases with either FAA or rIL-2 alone was completely ineffective, whereas treatment with FAA plus rIL-2 was able to significantly prolong mean survival time ($p < 0.001$) from 38.7 days to 64.1 days with 30% of the mice rendered disease free. These results demonstrate that FAA plus rIL-2 is effective in treating Renca in various anatomical sites.

**Determination of optimal dose of FAA for combined modality therapy of Renca.** Initial experiments demonstrating therapeutic synergy between FAA and rIL-2, performed using high doses of FAA (200 to 250 mg/kg), demonstrated that the optimum dose range of rIL-2 was from 10,000 to 30,000 U/mouse (17). Additional experiments (Fig. 5) were performed to determine the optimum dose of FAA required for therapeutic responsiveness. Mice were treated with various doses of FAA and then administered the optimal daily dose of 30,000 U of rIL-2 (17). FAA doses of 150 mg/kg or more exhibited maximal therapeutic synergy [40 to 50% survival at all doses $\geq 150$ mg/kg] with rIL-2, whereas lower doses had virtually no effect. This dose response coincides closely with that previously presented for augmentation of NK activity (17) in normal mice and for optimal antitumor activity against other solid tumors (15).

**Contribution of T lymphocytes to therapeutic effects of FAA plus rIL-2.** Overall, our data demonstrate that there is a close correlation between augmentation of NK activity and the therapeutic effect of FAA plus rIL-2. We have also shown that in vivo treatment of renca-bearing mice with anti-asGM₁ serum greatly decreased the ther-
The results shown in Table II demonstrate that 71% of mice previously cured of residual Renca by FAA plus rIL-2 treatment were resistant to a subsequent intra-dermal rechallenge with Renca, whereas none of the normal mice similarly challenged with Renca survived. Thus, there is evidence that the therapeutic effects of FAA plus rIL-2 have contributions from both NK cells and T cells.

**Table II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Survivors/ No. of Re-Challenged</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>FAA plus rIL-2</td>
<td>10/14</td>
<td>71</td>
</tr>
</tbody>
</table>

* Mice cured of renca from two separate experiments with FAA plus rIL-2 therapy were re-challenged with $1 \times 10^6$ Renca cells s.c. 150 to 170 days after the initial tumor challenge.

**Table III**

<table>
<thead>
<tr>
<th>Hours Post-FAA Injection</th>
<th>Serum IFN (U/ml)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>8</td>
<td>4000</td>
</tr>
<tr>
<td>16</td>
<td>1000</td>
</tr>
<tr>
<td>24</td>
<td>360</td>
</tr>
<tr>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mice were injected with FAA (250 mg/kg split equally i.v. and i.p.). Blood was pooled from three mice in each group prior to serum harvest. Animals were bled for only one time point. These data are from a representative experiment which has been performed with similar results on three separate occasions.

The results shown in Table III demonstrate that a dose of FAA (250 mg/kg) which routinely exhibits therapeutic activity when administered in combination with rIL-2 also rapidly induces high levels of serum IFN (4000 U/ml by 4 h). Significant amounts of IFN can be detected up to 24 h after the injection of FAA. Additional studies have shown that 1) the type of IFN induced is α/β, 2) the induction of IFN is dose dependent and correlates closely with the therapeutic dose response, 3) that peak levels of IFN are induced 3 to 6 h after FAA administration, and 4) that levels of IFN induced by FAA are similar to those induced by a well known inducer of IFN poly ICLC, the complex of polyinosinic-polycytidylic acid and poly-L-lysine (33). These results suggest that the induction of IFN-α/β by FAA may contribute to the therapeutic efficacy of FAA when used in combination with rIL-2.

Treatment of Renca by rIFN-α A/D Bgl and rIL-2. Inasmuch as FAA rapidly induces high levels of IFNα/β (Table III), and clinical trials with rIFN-α have demonstrated some therapeutic activity against human renal cancer (34) we speculated that rIFN-α plus rIL-2 would have therapeutic activity against Renca. The experiment shown in Figure 6, performed to mimic the early induction of IFN-α/β by FAA followed by rIL-2 therapy, confirms that this combination of cytokines is moderately effective against murine renal cancer, though less so than FAA plus rIL-2. Administration of five 30,000 U doses of rIFN-α A/D Bgl during the 24 h immediately before the initiation of rIL-2 administration, in conjunction with a 6th dose of 30,000 U of rIFN-α A/D Bgl at the time of the 2nd dose of rIL-2, resulted in long term survival of 20% of the Renca-bearing mice, and a significant extension ($p < 0.001$) of the mean survival time to 60 days. In contrast, treatment with rIFN-α A/D Bgl alone did not result in any long term survivors, nor did it significantly increase survival time over that of untreated controls. Previous results have confirmed that rIL-2 alone has no activity (Fig. 2). Thus, these results support the conclusion that the mechanism by which FAA cooperates with rIL-2 for therapy of Renca is partially mediated through induction of IFN-α/β.

**Discussion**

Most pre-clinical (1-3, 5-7, 9-11) and clinical (4, 8) studies to date have used rIL-2 as a single agent or in combination with adoptive transfer of LAK cells. The
Impetus for these experiments has been largely provided by recent observations on the potent immunomodulatory capabilities of rIL-2 (1, 19–22). The widespread application of rIL-2 to cancer treatment has been partially hindered by toxicity induced by high doses of rIL-2 (12), as well as the expense and logistical difficulties in performing AIT in association with rIL-2. Thus, approaches to cancer treatment which exploit the use of rIL-2 in situations that require less than maximal doses of rIL-2, and provide alternatives to the use of AIT would increase the practicability of rIL-2 as an anti-tumor agent. One such approach would be to combine rIL-2 with other BRM or with chemotherapeutic drugs with the expectation that such combinations may exhibit enhanced anti-tumor activity. Inasmuch as the investigational drug FAA potently stimulates NK cells (17, 18), and NK cells mediate antimetastatic effects (35, 36) and contribute much of the LAK activity generated by the culture of lymphocytes in rIL-2 (19−22), the present studies were designed to determine the efficacy of murine renal cancer treatment in mice with FAA plus rIL-2.

The present studies demonstrate that FAA and rIL-2 potently augment NK activity in Renca-bearing mice in both lymphoid and nonlymphoid organs (Fig. 1; Table 1), and synergize to provide enhanced therapeutic effects against renca. FAA plus rIL-2 rendered approximately 60% of all treated mice disease free (Fig. 2). This success rate compares favorably to that previously reported for this tumor model using adoptive chemoimmunotherapy by doxorubicin hydrochloride and AIT (9, 13). However, the effects of FAA + rIL-2 are conspicuous by the lack of a requirement for AIT. Furthermore, subsequent experiments demonstrated that the addition of AIT by rIL-2-stimulated lymphocytes did not improve the efficacy of the FAA plus rIL-2 combination (Fig. 2). An additional benefit derived from using rIL-2 in combination with FAA is the ability to use less toxic doses of rIL-2 (17) than those used in rIL-2 alone (2, 4, 7) or FAA + rIL-2 protocols (3, 7, 8). Similarly, toxicity due to FAA in this combined approach is also limited because therapeutic efficacy (Fig. 5) is observed at FAA doses well below the LD10 for this drug.

There are several possible mechanisms by which FAA could enhance the anti-tumor efficacy of rIL-2. FAA might exert direct anti-tumor cytotoxicity and thereby reduce the tumor burden sufficiently to allow rIL-2-induced immune augmentation to successfully control the residual tumor. This seems unlikely because FAA is largely ineffective in inhibiting the growth of Renca in vitro (T. Sayers, T. A. Wiltrait, C. Hustead, K. McCormick, and R. H. Willtrout, manuscript in preparation). However, this result does not preclude the possibility that cytoreduction could occur via toxic metabolites of FAA. This possibility is currently under active investigation in several laboratories. Alternatively, FAA may either directly or indirectly stimulate the immune system such that the subsequent administration of rIL-2 results in a synergistic anti-tumor response. This hypothesis is supported by the observation that FAA plus rIL-2 stimulates NK activity to a significantly greater degree than does either FAA or rIL-2 alone (Table 1). Although it is tempting to speculate that such enhanced cytolytic activity could directly contribute to enhanced anti-tumor activity, it is important to note that NK cells are also potent immunoregulatory cells in that they synthesize and secrete a variety of biologically active immunomodulatory molecules, including IL-1, IL-2, IFN-α, IFN-γ, and TNF (31). Any of these molecules might synergize with rIL-2 or rIL-2-induced cytokines for an enhanced anti-tumor response. Other mechanisms by which FAA might enhance the effectiveness of rIL-2 include the removal of suppressor T cells or the induction of tumor associated Ag. The removal of suppressor cells seems an unlikely mechanism because the use of cyclophosphamide at doses that have been shown to eliminate or reduce immunosuppression (6, 37), did not enhance the effectiveness of rIL-2, suggesting that the anti-tumor activity associated with FAA may not be mediated by removal of immunosuppression (data not shown).

We have examined several potential mechanisms by which FAA could act synergistically with rIL-2. First, FAA + rIL-2 augments NK activity to a greater degree than does FAA or rIL-2 alone. This suggests that NK cells may play a role in the antitumor effects induced by FAA + rIL-2. The effectiveness of FAA + rIL-2 against Renca was substantially reduced but not eliminated by administration of anti-asGM, serum (17). This supports a role for NK cells in the therapeutic effect but it is not completely conclusive because other cell types, including macrophages (38, 39) and certain populations of T lym-
phocytes (40, 41) can express the asialo GM,
Ag, although the function of macrophages and T lymphocytes appears to be less sensitive to the in vivo effects of this antiserum than is the function of NK cells (38, 41). Future studies in this area will require the selection of NK-specific antibodies that selectively eliminate murine NK cells in vivo. Unfortunately, such antibodies are not currently available for BALB/c mice.

Another possible mechanism for the synergistic anti-tumor effects of FAA plus rIL-2 would be the induction of cytokines such as IL-1, IFN-α/β, or TNF which could synergize with IL-2 or IL-2-induced cytokines like IFN-γ. There have been reports that IFNa and IL2 can act synergistically for enhancement of NK activity (42), as well as for therapy of the M5076 reticulum cell sarcoma (43) in mice. Similarly, the combination of IL-1 and IL-2 has been shown to enhance proliferation of human NK and T cells (44). Inasmuch as IL-2 has been shown to induce the production of IFN-γ from NK cells (45, 46) and T lymphocytes (47), it is conceivable that FAA-induced cytokines could interact with IFN-γ to generate synergistic anti-tumor effects. In fact, previous studies have shown that TNF can synergize with IFN-γ for treatment of B16 melanoma in mice (48) and that IFN-α/β can synergize with IFN-γ for antiproliferative activity against mouse (49) and human (50) tumor cells. Our studies demonstrate that administration of FAA rapidly induces the production and release of large amounts of IFN into the serum. Subsequent studies have demonstrated that the activity of this serum IFN activity is completely neutralized by anti-α/β antibodies and molecular studies have detected enhanced IFN-α mRNA expression within 3 h of in vivo administration of FAA in mice (33).

The IFN-α/β induced by FAA synergizes with rIL-2 for therapy of murine renal cancer (Fig. 6) because combined administration of human rIFN-α A/D Bg1 and rIL-2 to renca-bearing mice significantly prolonged survival. There are several possible mechanisms by which IFN-α/β (induced by FAA) could synergize with rIL-2 for anti-tumor effects. First, there is evidence that IFN-α and IFN-γ can synergize for augmentation of NK activity (51). This observation could explain the enhanced augmentation of NK activity observed after treatment of FAA plus rIL-2 (Table I), inasmuch as FAA could induce IFN-α and rIL-2 could induce IFN-γ, perhaps by production from NK cells. Alternatively, various combinations of cytokines could exhibit direct anti-proliferative effects. Preliminary studies from our laboratory have noted that proliferation of renca is partially inhibited by human rIFN-α A/D Bg1 in vitro and that this antiproliferative effect is markedly enhanced by rIL-2 (T. Sayers, T. A. Wiltrout, C. Hustead, K. McCormick, and R. H. Wiltrout, manuscript in preparation).

Thus, IFN-α/β induced by FAA may have some direct effects of Renca growth in vivo. Recent clinical observations describing responses of cancer patients to IFN-α therapy (52–54) are consistent with this result. However, it seems more likely that the combination of IFN-α with IL-2 or IL-2-induced cytokines, such as IFN-γ, are probably necessary because FAA alone exhibits little therapeutic activity in our model and the human rIFN-α A/D Bg1 plus rIL-2 is more effective than human rIFN-α A/D Bg1 alone (Fig. 6).

However, the anti-tumor effects of human rIFN-α A/D Bg1 plus rIL-2 do not fully match those induced by FAA plus rIL-2. Furthermore, the therapeutic efficacy of poly ILC plus IL-2 is not as great as FAA plus IL-2 (R. L. Hornung, unpublished observation), yet poly ILC and FAA are comparably potent IFN inducers (33). Therefore, it seems unlikely that the only contribution of FAA is the induction of IFN-α/β production. It seems more likely that FAA has pleiotropic effects, perhaps including the induction of other cytokines, such as IL-1 or TNF. There is already suggestive evidence that FAA may induce TNF (55). In addition to the participation of induced cytokines, and perhaps directly related to it, there is evidence that T cells may be involved in the antitumor effects of FAA and IL-2. Many mice cured of renca by treatment with FAA plus rIL-2 are resistant to subsequent re-challenge with renca (Table II). These results are in contrast to our previous data with the use of doxorubicin plus LAK cells plus rIL-2 in this model, where mice cured of renca were not immune to re-challenge (9, 13). Additional studies have also shown that FAA plus rIL-2 was largely ineffective in athymic mice. Thus it appears that the T lymphocyte compartment of the immune system plays a supporting role in the success of the FAA plus rIL-2 combination therapy. Thus, T lymphocytes probably contribute to anti-tumor therapy in this model both by production of cytokines in response to FAA and/or rIL-2, or by their development as tumoricidal effector cells. Current studies are focused on determining whether the resistance to rechallenge is tumor specific and whether cytolytic T cells are induced in Renca-bearing mice after treatment with FAA plus rIL-2. There is also evidence that IFN-α (56) can increase the expression of surface Ag on tumor cells. Such a mechanism could account for an induction of tumor-associated Ag that could allow for the generation of a beneficial T cell-mediated response, perhaps under stimulation by IL-2.

Overall, the results presented herein demonstrate that FAA plus rIL-2 is an effective treatment for murine renal cancer. The results suggest that this effect is partially mediated by the ability of FAA to induce IFN-α/β, and probably other cytokines, which synergize with rIL-2 or rIL-2-induced cytokines for enhanced therapeutic effects. The results also suggest that both NK cells (17) and T cells are required for optimal antitumor activity by FAA plus rIL-2. Finally, this model demonstrates that moderate doses of rIL-2 can have potent anti-tumor effects in the absence of adoptively transferred cells when combined with other drugs or cytokines.

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