ABSTRACT

Flavone acetic acid (FAA) is an investigational drug that augments natural killer activity, induces the genes for α- and γ-interferon (IFN) and tumor necrosis factor α, and synergizes with recombinant interleukin 2 for the successful treatment of murine renal cancer. However, in most clinical studies of FAA only minimal immunomodulatory effects have been reported. Most of the patients in these studies have also been given sodium bicarbonate to prevent possible nephrotoxicity. The current study was performed to determine whether alkalization had any effects on FAA-induced immune modulation and therapeutic activity in mice. The results showed that alkalization inhibited the treatment of murine renal cancer by FAA plus recombinant interleukin 2 such that the survival rate of 84% in nonalkalinized mice was reduced to 0 in mice that were alkalitized during treatment. Alkalization also significantly inhibited the ability of FAA to augment both splenic and hepatic natural killer activity in a dose-dependent manner. In contrast, alkalization did not inhibit the ability of polyinosinic-polyribocytidylic acid and poly-L-lysine stabilized in carboxymethyl cellulose, maleic anhydride divinyl ether, or Propionibacterium acnes to augment liver-associated natural killer activity. By Northern blot analysis, it was shown that the induction of mRNA for IFN-α, IFN-γ, and tumor necrosis factor α by FAA in the spleen cells of mice was significantly reduced in alkalinitized mice. Consistent with a reduction in the FAA-induced expression of the cytokine genes, alkalization also resulted in a significant decrease in both the peak serum concentration and duration of detectable IFN activity following FAA treatment. Increasing the dose of FAA in alkalinitized mice to 300 mg/kg overcame the deleterious effects of alkalization for treatment of murine renal cancer by FAA plus recombinant interleukin 2. These results demonstrate that the process of alkalization inhibits the immunomodulatory and immunotherapeutic effects of FAA in mice and suggest that alkalization might have similar deleterious effects on FAA-induced immune stimulation in human clinical trials.

INTRODUCTION

FAA is a synthetic flavonoid compound that has pleiotropic biological and antitumor activities (1, 2). This compound was originally found to be active against the murine colon adenocarcinoma 38 which is refractory to many conventional antitumor drugs (3). Further preclinical studies showed that FAA had some antitumor activity against a broad spectrum of murine solid tumors including colon carcinoma, pancreatic ductal adenocarcinoma, mammary adenocarcinoma, reticulum cell sarcoma, and Glasgow's osteosarcoma (4). It was also reported that FAA might have indirect antitumor activities. FAA has been shown to augment systemic NK activity in normal and tumor-bearing mice (5, 6) as well as in the peripheral blood of some cancer patients (7). Studies in our laboratory showed that the combination of FAA plus rIL2 had synergistic antitumor effects against an established Renca in BALB/c mice, while FAA or rIL2 administration alone did not produce significant antitumor effects (8). We have also observed that this synergistic antitumor effect of the combination of FAA plus rIL2 correlated with both the augmentation of NK activity by FAA (8) and induction of various cytokines, including IFN-α, and γ and TNF-α as shown by bioactivities and upregulation of specific mRNA (9).

Based on the preclinical observations, several Phase I clinical trials using FAA plus rIL2 were designed. Since FAA is relatively insoluble at a neutral or acidic pH, most clinical studies of FAA have used alkalization of the urine via sodium bicarbonate infusion before FAA administration to prevent potential nephrotoxicity caused by the crystallization of FAA at acidic pHs in the renal tubules (10–12). The studies reported herein demonstrate that the preclinical therapeutic efficacy of the combination of FAA plus rIL2 for Renca, as well as the immunomodulatory effects of FAA, was significantly reduced by systemic alkalization. These results suggest that the antitumor effects of FAA plus rIL2 in vivo are mediated partially by FAA-induced cytokines and demonstrate that the process of alkalization can impair the immunomodulatory and immunotherapeutic effects of FAA.

MATERIALS AND METHODS

Mice. Male 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 adenocarcinoma of spontaneous origin (13) was kindly provided by Dr. E. J. Pontes and Dr. G. P. Murphy (Roswell Park Memorial Institute, Buffalo, NY) and maintained in BALB/c mice by serial i.p. passage. YAC-1, a Moloney virus-induced lymphoma of A/SN origin, was used for assessment of NK activity. This cell line was maintained in suspension culture in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) with 10% fetal bovine serum (Sterile Systems, Logan, UT), 100 units/ml of penicillin (GIBCO Laboratories, Grand Island, NY), 100 μg/ml of streptomycin (GIBCO), and 2 mM L-glutamine (GIBCO). All tumor cell lines were shown to be free of Mycoplasma as well as pneumonia and minute virus of mice, mouse hepatitis and adenovirus, and Sendai, echoviria, lymphocytic choriomeningitis, and lactic dehydrogenase-elevating viruses (Animal Health Diagnostic Laboratory, NCI-Frederick Cancer Research Facility).
Reagents. FAA was synthesized by Lyonnaise Industrielle Pharmaceutique (LIPHA, Lyon, France) and obtained from the Pharmaceutical Resources Branch, NCI, Bethesda, MD. PolyICLC was kindly provided by Dr. Hilton Levy (National Institute of Allergy and Infectious Diseases, NCI-Frederick Cancer Research Facility, Frederick, MD) and used i.v. at a dose of 10 µg/mouse. Propionibacterium acnes was obtained from Burroughs Wellcome, Inc. (Research Triangle Park, NC) and used i.p. at a concentration of 35 mg/kg. The pyran copolymer MVE-2 was obtained from Adria Laboratories (Columbus, OH) and used i.v. at a concentration of 25 mg/kg. Cetus Corp. (Emeryville, CA) generously supplied rhIL2 in lyophilized form.

Cell-mediated Cytotoxicity Assay. NK activity was assessed using effector leukocytes obtained from spleen or liver as previously described (5, 8, 14). Various numbers of effector cells were incubated in triplicate with 1 x 10^4 3HCr-labeled YAC-1 tumor targets in round-bottomed, 96-well microtiter plates at 37°C for 4 h in RPMI 1640 medium supplemented with 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 µg/ml), L-glutamine (2 mmol), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (20 mmol). Supernatants were then removed from the wells, using the Titertek harvesting system (Flow Laboratories, McLean, VA), and counted on a Beckman Gamma 5500 radiation counter (Beckman Instruments, Inc., Irvine, CA).

% of specific cytotoxicity =

\[
cpm \text{ released from test well} - cpm \text{ released from target cells alone} \\
\text{total solubilized cpm} - cpm \text{ released from target cells alone}
\]

Data are expressed as lytic units_50 (LU_50), where 1 LU is the fraction of effector cells required to lyse 20% of 1 x 10^5 target cells.

Experimental Model. The Renca carcinoma was maintained by continuous i.p. passage in BALB/c mice. Harvest and inoculation of tumor cells were performed as previously described (15, 16). Briefly, intraperitoneal tumor nodules were excised under sterile conditions from Renca-bearing mice, and single cell suspensions were prepared by mechanical dissociation and filtration of the resulting tumor cells through gauze. Intrarenal tumor inoculations were routinely performed by injecting 1 x 10^6 tumor cells in 0.1 ml of HBSS (Mediatech, Inc., Herndon, VA) under the capsule of the left kidney. For some studies, the primary tumor mass was surgically removed by nephrectomy 11 days after Renca implantation. Within several hours of nephrectomy, mice were given a single bicompartamental (50% of the dose i.v. and 50% of the dose i.p.) administration of FAA. In other studies, treatment was initiated on Day 7 with the primary tumor in place. One day after FAA administration, mice received the first of 4 daily i.p. injections of 30,000 units of rIL2. Tumor progression and survival were monitored on a daily basis. Mice that appeared moribund were euthanized and examined for the presence of tumor.

Alkalization. Mice were given injections i.p. with various amounts of 5% sodium bicarbonate (NaHCO_3) 1 h prior to administration of FAA. The urinary pH of the mice was confirmed to be between 7.8 and 8.0 immediately prior to FAA administration. The pH of the urine was monitored using pocket indicator pHial paper (EM Reagents, Cherry Hill, NJ) every 30 to 60 min for 8 h after administration of FAA. When the pH decreased to 7.0, an additional 0.5-ml injection of NaHCO_3 was administered i.p. In most mice a repeat injection of NaHCO_3 was not required until 4 to 5 h after the administration of FAA.

Cytokine Assays. Serum IFN activity was determined using the vesicular stomatitis viral inhibition assay (17) with 1 unit of IFN being equal to the amount of IFN in 1 ml of sample that reduces viral lysis 50% of the time. The concentration was determined by measurement at A_560.

Northern Blotting Analysis. Total RNA (10 µg) was denatured by heating for 10 min at 65°C and then electrophoresed in a 1% agarose gel containing 6.5% formaldehyde for 5 h at 60 V. Separated mRNA was transferred to a nylon filter membrane (Rothm, Arlington Heights, IL) by capillary action using 20x SSC (Digene Diagnostic, Inc., Silver Spring, MD). The membrane was baked at 85°C for 2 h under vacuum and prehybridized at 42°C overnight in prehybridization solution (Hibrisol 1; Oncol, Gaithersburg, MD). The cdNA probe for murine TNF-α was generously provided by Dr. Anthony Cerami (Rockefeller University, New York, NY); the cdNA probe for murine IFN-γ was kindly donated by Dr. Ken-ichi Arai (DNAX Corp., Palo Alto, CA), and the cdNA probe for IFN-α was generously provided by Dr. Paula Pitha-Rowe, Johns Hopkins University Cancer Center, Baltimore, MD. These probes were labeled with deoxycytidine [5′-32P]-triphosphate triethylammonium salt (3000 Ci/mmole) utilizing a random primer DNA-labeling kit (Boehringer Mannheim Biochemical, Indianapolis, IN). Unincorporated [α-32P]dCTP was removed by a nucleic acid column chromatography system (Bethesda Research Laboratories, Bethesda, MD). Hybridization was performed at 42°C overnight in the above prehybridization solution supplemented with a 32P-labeled cdNA probe at 1 x 10^4 cpm/ml. After hybridization, the membrane was washed twice with 2x SSC and 0.1% SDS at room temperature for 10 min and then in 0.2x SSC and 0.1% SDS at 50°C for 10 min. The membrane was exposed to X-ray film (XOMAT film; Kodak, Rochester, NY) with an intensifying screen at ~70°C for various periods of time. After exposure, all filters were stripped twice by boiling in 0.1% SDS and 0.01x SSC for 20 min and rehybridized with chicken β-actin cdNA (kindly provided by Dr. D. W. Cleveland, Johns Hopkins University, Baltimore, MD). Lanes within each blot were found to hybridize to this cdNA with approximately equal intensity, indicating that comparable levels of RNA were present in each lane (data not shown).

Statistical Analyses. Differences in long-term survival duration between various treatment groups were analyzed using Student's t test. Statistical analyses of changes in NK activity were performed using Student's t test.

RESULTS

Effects of Alkalization on the Treatment of Established Renca by FAA in Combination with rIL2. We have previously demonstrated that the combination of FAA plus rIL2 was more effective than either FAA or rIL2 alone for the treatment of established Renca (5, 8). Since clinical trials of FAA plus rIL2 differ from the murine model in that patients are routinely alkalized, we performed studies to determine if alkalization impacts on the therapeutic efficacy of FAA plus rIL2 for Renca in mice. The results shown in Fig. 1 demonstrate that alkalization significantly decreased (P < 0.01) the incidence of long-term survivors induced by FAA plus rIL2. Renca-bearing mice treated with FAA plus rIL2 exhibited an 84% incidence of long-term disease-free survival, while the same treatment performed in alkalized mice resulted in no survivors. Control Renca-bearing mice treated only with HBSS in the presence or absence of alkalization routinely died of their disease as expected. Therefore, alkalization of Renca-bearing mice proved detrimental to the successful use of FAA plus rIL2 for treatment of this murine renal cancer.
SYSTEMIC ALKALINIZATION, FAA, AND MURINE RENAL CANCER

Fig. 1. Inhibitory effects of alkalinization on the treatment of Renca-bearing mice by FAA plus rIL2. BALB/c mice were given injections of 1 x 10^6 Renca cells under the capsule of the left kidney, and the tumor-bearing kidney was surgically removed 11 days later. Mice bearing residual tumor were then treated with a single administration of FAA (250 mg/kg) 6 h after removal of the tumor-bearing kidney on Day 11, followed by daily i.p. doses of rIL2 (30,000 units/day) on Days 12 to 15. Some mice in each treatment group were alkalinized prior to and during the FAA administration, as described in "Materials and Methods." Alkalinization significantly (P < 0.01) reduced the incidence of long-term survivors following treatment with FAA plus rIL2.

Table 1 Effects of alkalinization on the ability of FAA to augment NK activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NK activity (LU/organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>16</td>
</tr>
<tr>
<td>FAA</td>
<td>398</td>
</tr>
<tr>
<td>FAA + NaHCO_3 (0.5 ml)</td>
<td>282*</td>
</tr>
<tr>
<td>FAA + NaHCO_3 (0.2 ml)</td>
<td>342</td>
</tr>
</tbody>
</table>

Table 2 Effects of alkalinization on the ability of various biological response modifiers to augment liver-associated NK activity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>NK activity (LU/liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HBSS</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>FAA</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>FAA + NaHCO_3</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>PolyICLC</td>
<td>254</td>
</tr>
<tr>
<td></td>
<td>P. acnes + NaHCO_3</td>
<td>226</td>
</tr>
<tr>
<td>2</td>
<td>HBSS</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>FAA</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>FAA + NaHCO_3</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>MVE-2</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>MVE-2 + NaHCO_3</td>
<td>115</td>
</tr>
</tbody>
</table>

Effects of Alkalinization on the Augmentation of NK Activity in Mice. Since previous studies have suggested that the immunomodulatory properties of FAA were related to its therapeutic efficacy for Renca when used in combination with rIL2 (5, 9), we compared the ability of FAA to augment NK activity in alkalinized and nonalkalinized mice. The results shown in Table 1 confirm that FAA augments NK activity in the spleen and the liver. However, alkalinization significantly decreased the FAA-induced augmentation of splenic NK activity from 398 LU to 282 LU (P < 0.05) and more profoundly decreased the liver-associated NK activity from 78 LU to 40 LU (P < 0.01). This effect, as well as the therapeutic effects of FAA plus rIL2 (Fig. 2), was dependent on the dose of NaHCO_3 administered since the use of 0.2 ml of NaHCO_3 did not significantly inhibit NK activity in either organ. Since alkalinization partially inhibited the augmentation of NK activity by FAA, we performed additional experiments to determine whether alkalinization impaired the ability of other biological response modifiers to augment liver-associated NK activity. The results shown in Table 2 confirm that alkalinization significantly inhibits the ability of FAA to augment liver-associated NK activity. However, the ability of polyICLC, P. acnes, and MVE-2 to augment liver-associated NK activity is not similarly impaired by alkalinization.

Effects of Alkalinization on Induction of Serum IFN Activity by FAA in Vivo. Previous studies from our laboratory have shown that FAA induced both IFN and TNF activities in the sera of the mice within 1.5 to 3 h (8, 9, 19). These studies also showed that the detection of biologically active proteins in the sera was preceded by a distinct upregulation in expression of the genes for TNF-α, IFN-α, and IFN-γ such that mRNA for the cytokines was detectable by Northern blot analysis within 1.5 h after FAA injection (9). Both cytokine induction by FAA (9) and therapeutic synergy with rIL2 are strictly dose dependent such that doses of FAA ≥ 150 mg/kg are active for both functions, while doses <150 mg/kg were essentially inactive (5, 8). These results further suggested a role for the cytokines induced by FAA in its therapeutic efficacy with rIL2. Since alkalinization inhibited the ability of FAA to augment NK activity and to synergize with rIL2 for the treatment of Renca, we speculated that cytokine induction would also be suppressed in alkalinized mice.

The results shown in Fig. 3 demonstrate that serum IFN activity at all times tested and peak serum levels of IFN induced by FAA were decreased by alkalinization. Peak FAA-induced serum IFN activity in alkalinized mice was reduced by 50% (1250 units/ml versus 2500 units/ml) as compared with nonalkalinized mice. Perhaps more importantly, serum levels of IFN activity returned to near baseline by 6 h (75 units/ml),...
while 1250 units/ml of IFN activity were detectable in the serum of nonalkalinized mice at this time. In nonalkalinized mice, serum levels of FAA-induced IFN activity remained elevated even at 24 h (350 units/ml). Therefore alkalinization decreased the peak levels of serum IFN activity attainable following FAA and greatly shortened the time course during which elevated IFN levels were detected.

Effects of Alkalinization on Induction of Cytokine Gene Expression by FAA. The results presented in Fig. 3 demonstrate that alkalinization decreased the levels of IFN activity in FAA-treated mice. However, it remained unclear as to whether this effect was due to an inhibition of IFN production or to an alteration in FAA pharmacokinetics or biodistribution. The study shown in Fig. 5 was designed to determine whether the increase in FAA-induced IFN activity could overcome the inhibitory effects of alkalinization for the treatment of Renca by FAA plus rIL2. Treatment of mice with FAA at the routinely used therapeutic doses of either 150 mg/kg or 200 mg/kg prior to rIL2 administration proved successful for regression of the Renca tumor, with 55% and 100%, respectively, of the mice becoming long-term survivors. As expected, alkalinization of these mice reduced the long-term survival incidences to 0 and 20%, respectively. In contrast, when the dose of FAA was increased to 300 mg/kg which is close to the maximum tolerated dose, alkalinization had no deleterious effect on the therapeutic efficacy of FAA plus rIL2 for Renca. These results suggest that the inhibitory effects of alkalinization in the mouse can be overcome by further dose escalation of FAA.

DISCUSSION

Although there have been several promising preclinical studies reporting antitumor effects of FAA (3–5), little immune modulation and no therapeutic efficacy have been reported in human clinical trials of FAA (7,10–12). One difference between the preclinical studies and most clinical trials has been the use of alkalinization to prevent crystallization of FAA in the renal tubules. In fact, the inability of FAA to induce IFN or augment NK activity in our own ongoing Phase I clinical trial of FAA plus rIL2 suggested that the process of alkalinization might be deleterious. The studies reported herein demonstrate that this process of alkalinization in Renca-bearing mice results in a loss of most FAA-induced immunomodulatory and immunotherapeutic activities. Specifically, the ability of FAA to induce cytokine gene expression for IFN-α, IFN-γ, and TNF-α was inhibited by alkalinization, as was induction of serum IFN activity. Further, alkalinization partially reduced the ability of FAA to augment NK activity and reduced therapeutic synergy of FAA with rIL2 for Renca. Our previous studies have shown that: (a) FAA administration induced serum IFN and TNF activities shortly after inducing an upregulation in mRNA for IFN-α, IFN-γ, and TNF-α in leukocytes isolated from the spleen and/or liver (9, 18); (b) the dose of FAA required for therapeutic efficacy with rIL2 (≥150 mg/kg) correlated closely with doses (≥150 mg/kg) which could induce IFN-α, IFN-γ, and TNF-α mRNA in vivo

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3 S. Creekmore and W. Urba, personal communications.
failure of FAA to exhibit immunomodulatory or immunotherapeutic effects in mice, then the effects in clinical trials may be even more pronounced since the clearance of FAA from the serum of humans is more rapid than in mice (20, 21). Therefore, a failure of FAA to exhibit immunomodulatory or immunotherapeutic activities in humans could be attributed to an inability to obtain or maintain critical serum levels of FAA. The use of alkalinization may serve to further exacerbate this problem.

An alternative explanation for the disparity between preclinical and clinical results with FAA could be differences in metabolism of the drug. There is some evidence that rodents and humans degrade FAA to different metabolites and that the major glucuronide metabolite in humans undergoes chemical rearrangement at mildly alkaline pH (21). Thus, alkalinization may increase metabolite formation or even alter the activity of a critical metabolite in humans. However, we have recently found that FAA can rapidly and directly stimulate cytokine gene expression in splenic leukocytes in vitro, suggesting that metabolism of FAA is not required for immunomodulation in mice.

Several possible approaches can be taken to overcome the adverse effects of alkalinization on the therapeutic and immunomodulatory effects of FAA. First, it may be possible to avoid the reduction of therapeutic efficacy of FAA through alkalinization simply by escalating the doses of FAA that are administered. Thus it might be possible to administer higher doses of FAA to alkalinized mice with less toxicity than that seen in nonalkalinized mice. In fact, preliminary studies reported herein (Fig. 5) demonstrate that dose escalation of FAA to near the maximally tolerated dose does overcome the effects of alkalinization. Given the more rapid clearance and metabolism of FAA in humans, it remains unclear whether critical FAA levels in the serum can be attained and maintained simply by dose escalation. Second, the postulated nephrotoxicity in humans might be prevented by more extensive patient hydration. A clinical dose-escalating study for FAA plus rIL2 is now proceeding at our institution in the absence of alkalinization.

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rIL2 combinations. Such an approach could circumvent both the issues of alkalinization and differences in metabolites. Finally, the adverse effects of alkalinization might be overcome by using analogues or derivatives of FAA that are more soluble at neutral or acidic pHs. We are currently investigating several such compounds for therapeutic synergy with rIL2 as well as for their ability to induce cytokines.

In summary, we have demonstrated that the therapeutic efficacy of FAA in combination with rIL2 for treatment of Renca, as well as the ability to induce immunoactive cytokines, was markedly reduced by the process of alkalinization. These findings suggest that the lack of immunomodulatory and therapeutic activities by FAA in clinical trials could be at least partially due to the undesirable effects of alkalinization.

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Systemic Alkalization Inhibits the Ability of Flavone Acetic Acid to Augment Natural Killer Activity, Induce Cytokine Gene Expression, and Synergize with Interleukin 2 for the Treatment of Murine Renal Cancer
