Folate-Immunoglobulin G as an Anticancer Therapeutic Antibody

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Folate receptor-α (FR) is a promising cellular marker for tumor-specific drug delivery. Conjugation of folic acid to therapeutic and imaging agents has been shown to enhance their delivery to FR (+) cancer cells in vitro and in tumor-bearing mice via an FR-mediated cellular uptake mechanism. In this study, immunoglobulin G (IgG) was conjugated to folate and evaluated as a therapeutic antibody against folate receptor (FR)-positive tumors. Murine IgG (mIgG) was conjugated to folate via an amide bond to yield folate-conjugated mIgG (f-mIgG) that contained an average of ∼2.6 folates per molecule. Selective uptake of f-IgG by FR (+) tumor cells was determined by fluorescence microscopy and by flow cytometry. Lysis of L1210JF cells by NK cells from murine donors was increased 1.4−9.0-fold at the effector:target (E:T) ratio of 25:1, relative to control mIgG. In mice bearing L1210JF tumors, f-mIgG was found to significantly inhibit tumor growth and to have prolonged the median survival time (MeST). Significantly, the antitumor efficacy of f-mIgG was greatly increased when combined with liposomal G3139, an 18-mer phosphorothioate oligonucleotide. In fact, the combination resulted in a 100% cure rate among the tumor-bearing mice. Injection of f-mIgG significantly increased serum INF-γ and IL-6 level in mice compared with mIgG and dramatically increased serum INF-γ and IL-6 level when combined with liposomal G3139. These results suggested that f-IgG, a novel immunotherapy agent, has potent activity as a therapeutic antibody to the FR-positive cancer, and the therapeutic activity is enhanced by immunomodulatory agents.

INTRODUCTION

Folate receptor-α (FR) is a promising cellular marker for tumor-specific drug delivery, because it is up-regulated in many types of human epithelial cancers. These include tumors arising in the ovary, lung, kidney, mesothelium, head and neck, and brain (*1*−*5*). Conjugation of folic acid to therapeutic and imaging agents has been shown to enhance their delivery to FR(+) cancer cells in vitro and in tumor-bearing mice via an FR-mediated cellular uptake mechanism (*6*−*8*) and are currently being developed in the clinic by Endocyte Inc. Some folate conjugates have shown significant antitumor activities as immunotherapeutic agents (*9*−*12*). For example, folate−fluorescein was shown to eliminate FR(+) tumors in fluorescein-immunized mice (*11, 12*). Moreover, co-administration of interleukin 2 (IL-2) and interferon-α (IFN-α) was shown to enhance the therapeutic efficiency of the folate−fluorescein conjugate (*11*). In other studies, anti-FR-α monoclonal antibodies such as Mov-18 (*13, 14*) and MORAb-003 (Farletuzumab) (*15*) have been reported to target ovarian cancer and have undergone clinical evaluation as therapeutic agents with promising initial results. In addition, a folate-conjugated anti-TCR antibody was shown to re-target cytotoxic T lymphocytes to FR(+) tumors resulting in significant therapeutic activity (*16*−*18*).

G3139 (oblimersen or Genasense) is an 18-mer phosphorothioate oligonucleotide that was designed as an antisense agent against antiapoptotic factor bcl-2 (*19*−*21*). G3139 contains 2 CpG dinucleotides (*22*−*24*) and has been shown to induce a potent cytokine response, presumably through activation of toll-like receptor 9 (TLR9) (*25, 26*). Liposomal encapsulation of G3139 has recently been shown by us to further enhance the immunomodulatory effect of G3139 (*27*).

The objective of this study was to evaluate the therapeutic efficacy of folate conjugated IgG (f-IgG) as a therapeutic antibody against FR(+) tumors. F-mIgG conjugates was synthesized and evaluated in vitro as well as in vivo. Furthermore, the combined effect of f-mIgG and liposomal G3139 was also examined.

EXPERIMENTAL SECTION

Materials. Murine IgG (mIgG) was purchased from Equitech-Bio Inc. (Kerrville, TX). Folic acid, fluorescein isothiocyanate (FITC isomer I), *N*,*N*′-dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). G3139 was synthesized by Alpha DNA (Montreal, CA). Phosphatidylcholine from egg yolk (EPC), 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol hydrochloride (DC-Chol), and methoxy-poly(ethylene glycol) (MW = 2000) distearoyl phosphatidylethanolamine (mPEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). All chemicals used were of analytical or HPLC grade. Recombinant IL-2 with specific activity of 1×10^6 IU/mL was obtained from Hoffmann-La Roche Pharmaceuticals (Nutley, NJ).

Cell Lines. KB and K562 cells were purchased from American Type Culture Collection (Rockville, MD). L1210JF cells were provided by Dr. Manohar Ratnam at University of Toledo (Toledo, OH). KB, L1210JF cells were grown in folate-

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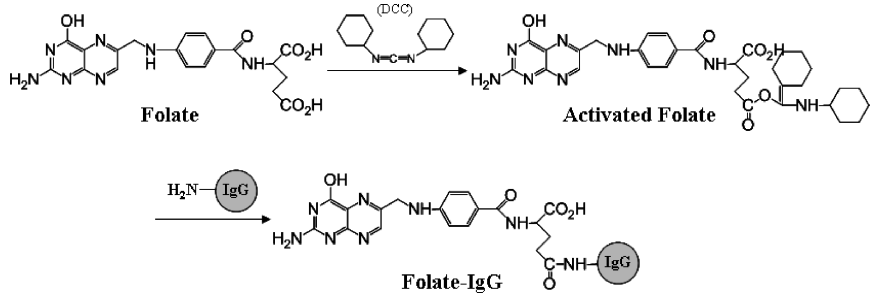

Figure 1. Synthesis of f-IgG conjugate. Conjugation to γ-carboxylic acid in folate is shown in this figure. However, the α-carboxylic acid group can also be conjugated.

free RPMI 1640 medium, and K562 cells were grown in RPMI1640 medium (Invitrogen Life Technologies Inc.). The media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

Synthesis of Folate-Conjugated Antibodies. The f-IgG was synthesized as previously described (*28*) with minor modifications. Briefly, folate was reacted with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) in DMSO at molar ratios of folate/DCC/NHS = 1/1.5/1.1 for 3 h at room temperature. The product f-NHS was centrifuged to remove byproducts and then was added to IgG in a pH 8.0 PBS buffer solution at a folate-to-IgG molar ratio of 12:1 for 2 h at room temperature. For the preparation of FITC labeled f-IgG (f-IgG-FITC) or IgG (IgG-FITC), 5× FITC dissolved in a small volume of DMSO was added to the protein solution at pH 8. To purify the conjugates, the reaction mixture was passed through a PD-10 column equilibrated in PBS (pH 8.0) and the product was collected in the void volume fractions. Folate content of the product was determined by UV spectrometry at 371 nm, and the antibody protein concentration was determined using the Pierce BCA Protein Assay Kit. A synthetic scheme is described in Figure 1.

Uptake of f-IgG-FITC by Tumor Cells. The cellular uptake of f-IgG was determined in FR(+) KB, L1210JF, and FR(-) K562 cells. Approximately 1×10^6 cells were incubated with 200 nM FITC labeled IgG or f-IgG in 1 mL folate-free RPMI1640 at 37 °C for 30 min. The cells were then washed three times with cold PBS containing 1% BSA and examined by fluorescence microscopy or by flow cytometry. For receptor blocking studies, 200 μM of free folate was added to media during f-IgG-FITC exposure.

Antibody Dependent Cellular Cytotoxicity (ADCC) Mediated by f-IgG in L1210JF Cells. Murine NK cells were isolated from splenocytes of DBA/2 mice (Charles River, Wilmington, MA) via mechanical digestion followed by lysis of red blood cells. Spleenocytes were then incubated with phycoerythrin-labeled anti-DX5 antibody (BD PharMingen, San Diego, CA) for 30 min at 4 °C. Following this, the cells were washed and incubated with antiphycoerythrin antibody-coated magnetic microbeads (Miltenyi Biotech, Auburn, CA) for an additional 30 min at 4 °C according to the instructions of the manufacturer. NK cells were isolated via positive selection through an LS+ magnetic column (Miltenyi Biotech). Isolated murine NK cells were >90% $DX5^+$ by FACS analysis. Purified murine NK cells were plated in 96-well V-bottom plates in 10% HAB containing glutamine free, folate-free RPMI 1640 medium supplemented with a low dose of IL-2 (1 nM), and incubated overnight at 37 °C. For ADCC assays, mIgG or f-mIgG treated, ^{51}Cr-labeled L1210JF tumor cells were added to NK cells at effector:target (E:T) ratios of 25:1 and 12.5:1 and 6.25:1, and cytotoxic interactions were allowed to proceed for 4 h at 37 °C. Target cell lysis was determined by counting 100 μL of supernatant in a scintillation counter after removal of intact cells by centrifugation. Spontaneous and maximum lysis were determined by incubating radiolabeled target cells in the absence of effector cells or by adding 10% SDS to the radiolabeled target cells, respectively. The percentage of cell lysis was calculated by the following formula: Lysis (%) = 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release).

Preparation of Liposomal G3139. Liposomes containing G3139 were prepared by the EtOH dilution/diafiltration method, as described previously (*27*). DC-Chol, egg PC, and mPEG-DSPE were dissolved in ethanol and mixed at a molar ratio of 30:65:5. The lipid mixture (20 mg/mL) was mixed with protamine solution in citrate buffer (pH 4.0, 20 mM) and then slowly added to G3139 solution in citrate buffer (pH 4.0, 20 mM) under vortex mixing to achieve a final ethanol concentration of 40% and a G3139, lipid, and protamine ratio of 1:12.5:0.3 (w/w/w). The preparation was then dialyzed against citrate buffer (pH 4.0, 20 mM) for 1 h and then against HEPES-buffered saline (HBS, pH 7.5) overnight to remove ethanol. Final product was stored at 4 °C before use. The mean particle size was determined by dynamic light scattering on a Nicomp model 370 Submicrometer Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). Zeta potential (ζ) of liposomal G3139 was measured on a Brookhaven 90plus Particle Analyzer (Holtsville, NY).

Therapeutic Efficacy in Murine Leukemia Model. Therapeutic efficacy in murine leukemia model was evaluated in DBA/2 mice (Charles River, Wilmington, MA). Female mice (18−22 g) were injected subcutaneously on day 0 with 1×10^6 L1210JF cells and then randomized to different treatment groups to eliminate cage effects. Before leukemia cell inoculation, mice were placed on a folate-free diet (AIN-90G, Dyets, Bethlehem, PA) for 1 week. Normal rodent chow was not used because it contains approximately 3.19 mg/kg folic acid, which leads to supra-physiological serum folate concentration. When a tumor reached a volume of ∼25 mm^3, the mice were treated with the desired immunotherapy regimens 4 times every 4 days (q4dx4). In the first treatment regimen, mice were given 100 mg/kg mIgG or f-mIgG intraperitoneally. In the second treatment regimen, mice were treated with 1 mg/kg free G3139 or liposomal G3139. In the third treatment regimen, mice were given 100 mg/kg f-mIgG combined with free G3139 or liposomal G3139. Tumor dimensions were determined by measuring with a caliper, and the tumor volume (in mm^3) was calculated by the formula 0.5 × (length × width × height). Mice were sacrificed once the tumor size reached greater than 1500 mm^3. Tumor inhibition ratio was calculated by $(C - T)/C \times 100\%$, where T and C represent the mean tumor volume of the treated and control animals, respectively. Animal survival was evaluated by Kaplan−Meier analysis and increase-in-

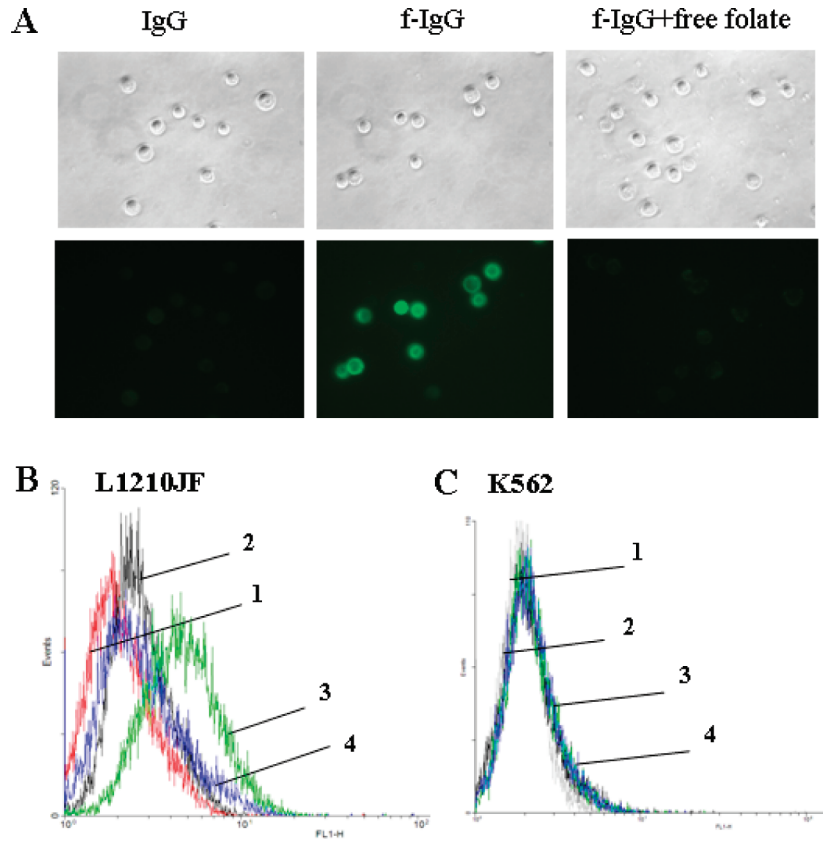

Figure 2. Uptake of f-IgG-FITC in KB (A), L1210JF (B), and K562 (C) cells. Cells were treated with 200 nM FITC labeled IgG or f-IgG in folate-free RPMI1640 at 37 °C for 30 min. For the receptor blocking study, 200 μM of free folate was added to media during f-IgG-FITC exposure. f-IgG-FITC binding in KB cells by fluorescence microscopy. After treatment, KB cells were photographed in both phase-contrast (upper panels) and fluorescence (lower panels) mode on a Nikon Eclipse 800 fluorescence microscopy. Uptake of f-IgG-FITC in L1210JF and K562 cells by flow cytometry. (1) Untreated cells. (2) Cells were treated with IgG-FITC. (3) Cells were treated with f-IgG-FITC. (4) Cells were treated with f-IgG-FITC and free folate.

lifespan (ILS, %) was calculated by ILS = (mean survival time of the treated mice/mean survival time of control mice − 1) × 100%.

Serum Cytokine Levels in Murine Leukemia Model. The serum cytokine levels were investigated in L1210JF tumor-bearing DBA/2 mice (Charles River, Wilmington, MA). Before leukemia cell inoculation, mice were placed on a folate-deficient diet (AIN-90G, Dyets, Bethlehem, PA) for at least 1 week. At 5 days after tumor cells inoculation, the mice were injected intraperitoneally with 100 mg/kg mIgG and f-mIgG. For the therapeutic combination study, f-mIgG was intraperitoneally administered with free G3139 or liposomal G3139. Blood samples were collected at 2, 6, and 24 h after injection. Serum was obtained by centrifugation of blood samples at 7000 × *g* for 10 min and stored −20 °C until the time of assay. IL-6 and INF-γ was determined by ELISA Ready-Set-Go kits.

Statistical Analysis. Statistical analysis of survival data was performed using the log-rank test. Comparisons between groups were made by Student's *t* tests using the MiniTAB software (Minitab Inc., State College, PA). Difference in survival or tumor growth were considered significant when the *p* value was <0.05.

RESULTS

Characterization of f-IgG Conjugates. The concentrations of IgG and folate were determined by BCA protein assay and UV spectrometry at 371 nm, respectively. Conjugation reactions performed at IgG-to-folate-NHS ratios of 12:1 and 100:1 yielded preparations containing an average molar ratio of 2.6 folates per IgG and 9.0 folates per IgG, respectively. Because excessive folate conjugation may adversely affect the effector function of the IgG, a lower conjugation level was desirable. Therefore, f-IgG with the lower conjugation level was selected for subsequent studies. The presence of folate on IgG was further confirmed by MALDI-TOF mass spectroscopy (data not shown).

To evaluate the FR targeting efficiency of f-IgG, tumor cell uptake of f-IgG-FITC was determined by fluorescence microscopy or by flow cytometry. In FR(+) KB cells, f-IgG-FITC was shown to be taken up much more compared to IgG-FITC (Figure 2A). The uptake of f-IgG-FITC was blocked by 200 μM free folate (Figure 2A). The results from flow cytometry were consistent with those obtained by fluorescence microscopy. In FR(+) murine L1210JF cells, incubation of f-IgG-FITC with cells resulted in greater fluorescence intensity compared with that of cells treated with IgG-FITC (Figure 2B). No increased fluorescence was observed when cells were incubated with f-IgG-FITC in the presence of 200 μM free folate (Figure 2B). In contrast, in FR(-) K562 cells, the uptake of f-IgG-FITC and of IgG-FITC were similar, and the uptake was not affected by 200 μM free folate (Figure 2C). These results indicated that f-IgG-FITC selectively targeted FR (+) tumor cells and was taken up via the FR.

F-IgG Enhanced NK Mediated ADCC against L1210JF Cells. The effects of f-IgG on NK cell lytic activity were examined in an ADCC assay using L1210JF tumor cells as targets. Murine NK cells were activated overnight in medium supplemented with IL-2 (1nM). These cells were then used as effectors against ^{51}Cr labeled target cells that were treated with either mIgG or f-mIgG. As shown in Figure 3A, f-IgG markedly enhanced the ADCC activity of NK cells at all E:T ratios

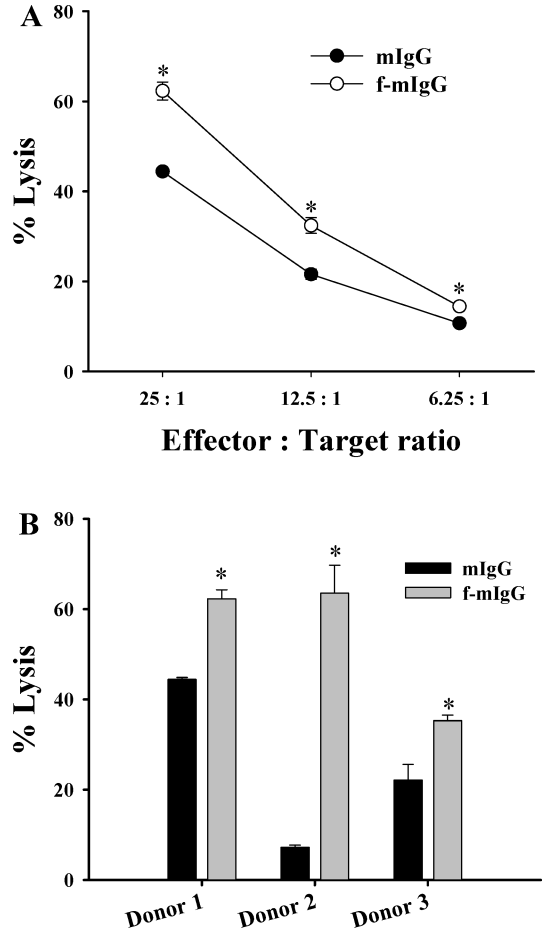

Figure 3. Effect of f-IgG on ADCC activity against FR-positive L1210JF cells at different effector:target ratio (A) and different donor from different mice (B). The NK cell lytic activity was assessed in a standard 4 h chromium release assay using f-mIgG-coated L1210JF cancer cells. The percentage of lysis was calculated as described in Experimental Section. Data represent the mean ± SD ($n = 3$).

examined compared with IgG (Figure 3A). Similar results was observed at E:T ratio of 25:1 for each of the three donors (Figure 3B). However, a relatively large variation was observed among different donors, presumably due to the variability in effector cell activities from different mice.

Characterization of Liposomal G3139. Liposomes containing G3139 was prepared by the EtOH dilution/diafiltration method. The G3139 liposomes showed mean diameter of 92.4 ± 43.6 nm, encapsulation efficiencies of >95%, and zeta potential of 5.02 ± 0.4 mV.

Therapeutic Activity of f-IgG in the Murine Leukemia Model. f-IgG was evaluated in the FR (+) L1210JF murine leukemia model. Mice bearing L1210JF tumors of ~25 mm^3 in size were treated with PBS, 100 mg/kg of mIgG, or 100 mg/kg of f-mIgG by intraperitoneal injection every 4 days for a total of 4 injections. The mice treated with f-mIgG had 59.6% reduction in tumor volume by day 24 after the engraftment of L1210JF cells compared with mIgG treated mice ($p < 0.05$) (Figure 4A). However, tumor-bearing mice treated with mIgG did not show any difference in tumor volume compared to the mice treated with PBS. The median survival time (MeST) for PBS, mIgG, or f-mIgG groups was 24, 27, and 39 days, respectively (Figure 4B, Table 1). Percentage ILS values are 62.5% for f-mIgG and 12.5% for mIgG. Moreover, 1 out of 5 mice was completely cured following treatment with f-mIgG (Figure 4B). The data indicated that f-IgG was more therapeutically effective than IgG or PBS in inhibiting tumor growth and prolonging animal survival ($p = 0.004\,01$; by log-rank test).

In order to investigate whether the anticancer activity was due to FR targeting, anticancer activity of f-mIgG was evaluated in the FR (-) L1210 murine leukemia model. As shown in Figure 4C, f -mIgG slightly decreased tumor volume by day 23 after the engraftment of L1210 cells compared with mIgG and PBS, although no significant differences were observed among f-mIgG, mIgG, and PBS. The median survival times (MeST) for PBS, mIgG, and f-mIgG were 25, 28, and 28, respectively. Similar to data on tumor inhibition, survival time was not significantly prolonged by treatment of f-mIgG compared with mIgG ($p = 0.535$; by log-rank test). Taken together, these results suggested that anticancer activity of f-mIgG in FR positive L1210JF tumor-bearing mice was likely due to the FR targeting.

Therapeutic Efficacy of Liposomal G3139 in Murine Leukemia Model. Previous studies have shown that the cellular uptake and the immunomodulatory effect of G3139 can be significantly enhanced when delivered in a liposomal form (*29*). In order to compare the antitumor efficacy of free G3139 and liposomal G3139, mice carrying L1210JF tumors were treated with 1 mg/kg of G3139 in free form or in liposomal form. As shown in Figure 5, tumor-bearing mice treated with liposomal G3139 had a 45.5% reduction in tumor volume compared with PBS-treated mice ($p < 0.05$). In contrast, free G3139 did not decrease tumor growth compared with PBS. The median survival times (MeST) for PBS, free G3139, or liposomal G3139 were 24, 36, and 36 days, respectively (Figure 5). However, there was no significant difference between free G3139 and liposomal G3139 ($p = 0.252$; by log-rank test). These data suggested that liposomal G3139 was more effective than free G3139 or PBS in mediating tumor regression but not in prolonging survival time of mice compared with free G3139.

Synergistic Effect of f-IgG Combined with Liposomal G3139. Loomis et al. (*30*) has shown that antitumor efficacy of G3139 significantly increased when combined with rituximab, a monoclonal antibody directed against CD20 in a SCID mouse xenograft model. Since f-IgG is being evaluated as a therapeutic antibody, we examined the antitumor efficacy of free and liposomal G3139 coadministered with mIgG or f-mIgG in the murine L1210JF leukemia model. Treatment of IgG combined with liposomal G3139 significantly increased MeST of the mice compared with the treatment of IgG combined with free G3139 (data not shown). With regard to tumor growth inhibition, liposomal G3139 further increased the antitumor efficacy of f-IgG (Figure 5A, Table 1). Importantly, all of the mice were rendered tumor-free after combined treatment with liposomal G3139 and f-IgG (Figure 5B). In comparison, less impressive 20% and 25% cure rates were observed following treatment with f-mIgG alone and following combined treatment with f-mIgG and free G3139, respectively (Figure 5B). These data indicate a strong synergistic effect between f-IgG and liposomal G3139.

Serum Cytokine Levels in Murine Leukemia Model. In order to investigate the effect of cytokine production on anticancer activity, the serum cytokine was determined in DBA/2 mice after administration of mIgG or f-mIgG and combined with free G3139 or liposomal G3139. As shown in Figure 6, levels of INF-γ peaked at 8 h, while levels of IL-6 peaked at 2 h. After 24 h, all cytokine levels were diminished. Compared with mIgG, f-mIgG showed significant cytokine (INF-γ and IL-6) production (Figure 6A,B). In combination with free G3139 and liposomal G3139, f-mIgG induced 2.3-fold and 6.1-fold higher INF-γ production than f-mIgG alone at 6 h postinjection, respectively. Similar results were observed from IL-6 induction in L1210JF murine leukemia model. These data suggested that increased uptake of f-mIgG might elevate the

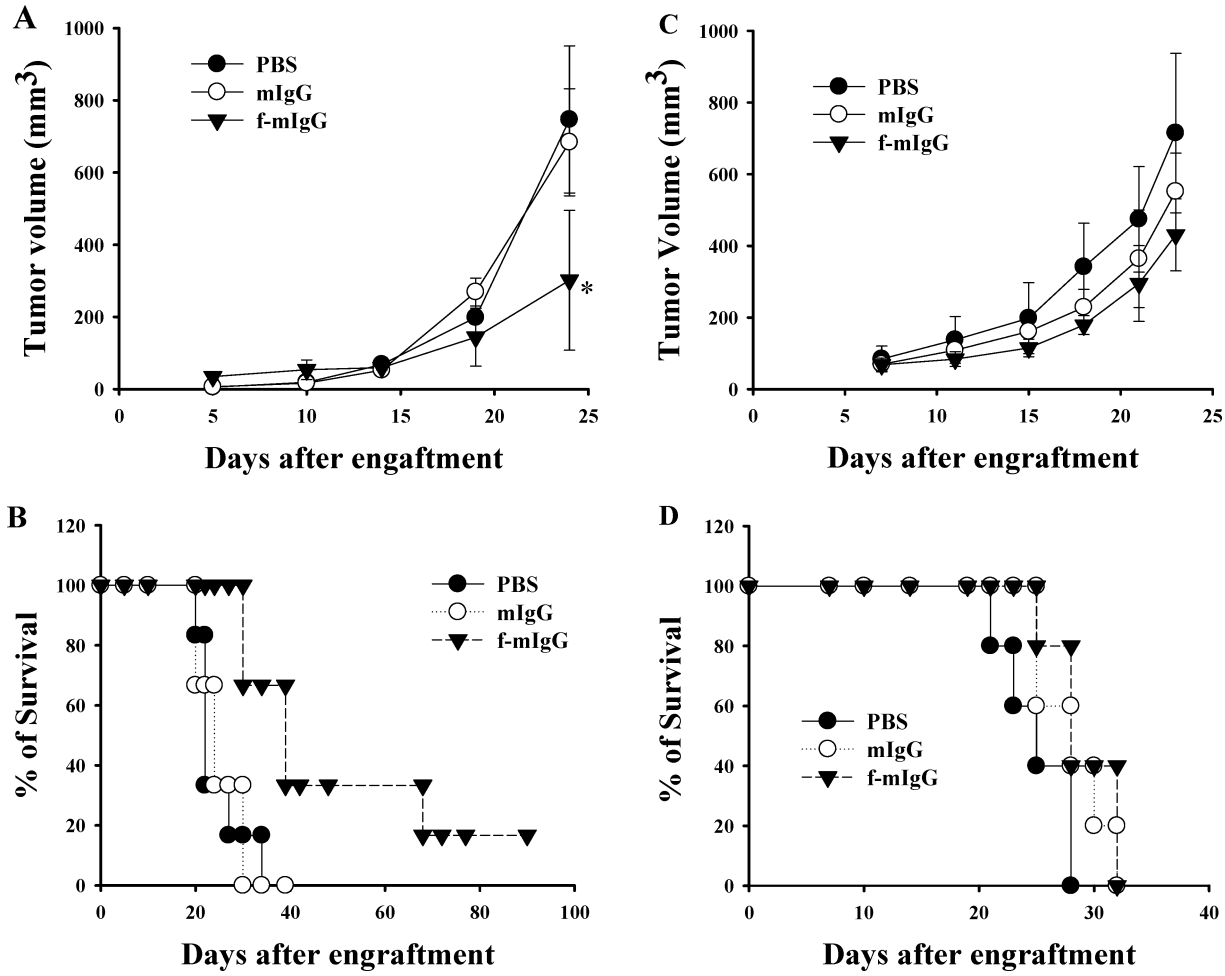

Figure 4. In vivo efficacy of folate receptor mediated immunotherapy in L1210JF (A,B) and L1210 (C,D) bearing DBA/2 mice. Mice (5−6 per group) were implanted subcutaneously on day 0 with 1×10^6 L1210JF cells. On day 5 or 7, animals were treated with 100 mg/kg of mIgG or f-mIgG. The treatment was continued every 4 days for four times. (A,C) Inhibition of tumor growth. Tumor dimensions were measured every 2 days using calipers. (B,D) Survival of L1210JF and L1210 tumor-bearing mice after treatment with mIgG or f-mIgG. PBS was injected as a vehicle control. (*, $P < 0.01$ compared with PBS group.)

Table 1. Survival of Mice after Treatment with mIgG and f-mIgG Alone or in Combination with Free or Liposomal G3139 (L-G3139) in L1210JF Tumor-Bearing Mice[a]

treatment	tumor inhibition ratio[b] (%)	MeST (days)	ILS[c] (%)	Log-rank *p* value
PBS	0	24	0	-
mIgG	8.4	27	12.5	0.901
f-mIgG	59.6	39	62.5	0.00401
G3139	5.6	36	50.0	0.0209
L-G3139	45.5	36	50.0	0.00804
f-mIgG+G3139	83.1	70	191.7	0.000495 (0.198)[d]
f-mIgG+L-G3139	100.0	∞	∞	0.000495 (0.00432)[d]

[a] $n = 6$ for each treatment group. [b] Tumor inhibition ratio was calculated by $(C - T)/C \times 100\%$, where T and C represent the mean tumor volume of the treated and control mice, respectively. [c] ILS(%) was calculated by (MeST of the treated mice/MeST of control mice − 1) × 100%, where MeST represents the mean survival time. [d] The *p* value was calculated in comparison to f-mIgG.

level of cytokines and combined with liposomal G3139 more potently increased the level of cytokines.

DISCUSSION

FR is a promising target for tumor-specific drug delivery. In this study, we describe a novel immunotherapy agent that combines the FR-targeting properties of folate and the effector functions of the IgG. Although folate−hapten's activities have been reported previously (*11, 12*), that strategy involves preimmunization of the animal and in vivo coupling of the folate−hapten and the endogenous antihapten antibody, which may present an challenging issue. The f-IgG strategy is more analogous to FRα monoclonal antibodies such as MOv-18 (*13, 14*) and Morab-003 (*15*) currently under clinical development. Compared with FRα monoclonal antibodies, f-IgG targets both FR subtypes, and the underlying IgG used is human and polyclonal, which may be less immunogenic.

f-IgG showed much stronger antitumor effect than IgG in the FR(+) L1210JF tumor-bearing mice (Figure 4A,B) but not in the FR(-) L1210 tumor-bearing mice (Figure 4C,D). These results presumably were due to FR-mediated tumor cell targeting. Tissue toxicity and splenomegaly was not founded by treatment of f-IgG in tumor-bearing mice. Selective folate receptor binding was also observed in the FR(+) L1210JF and KB cells in vitro (Figure 3). Besides folate receptor binding, Fc receptor (FcR) is critical in f-IgG mediated immunotherapy (*31*). FcR-expressing immune cells that recognize a constant (Fc) region of IgG and induce immune response such as ADCC, phagocytosis, neutrophil activation, and the paradoxical inhibition of B cell activation (*31−34*). The potential role of NK cell-mediated ADCC of f-IgG was examined. The results showed that lysis of L1210JF tumor cells by NK cells was increased 1.4−9.0-fold by treatment of f-IgG as compared with IgG (Figure 3). In contrast, f-IgG did not bind to FR(-) cell lines (e.g., A549 human alveolar basal epithelial) and did not enhance their lysis by NK cells (data not shown). These results suggested

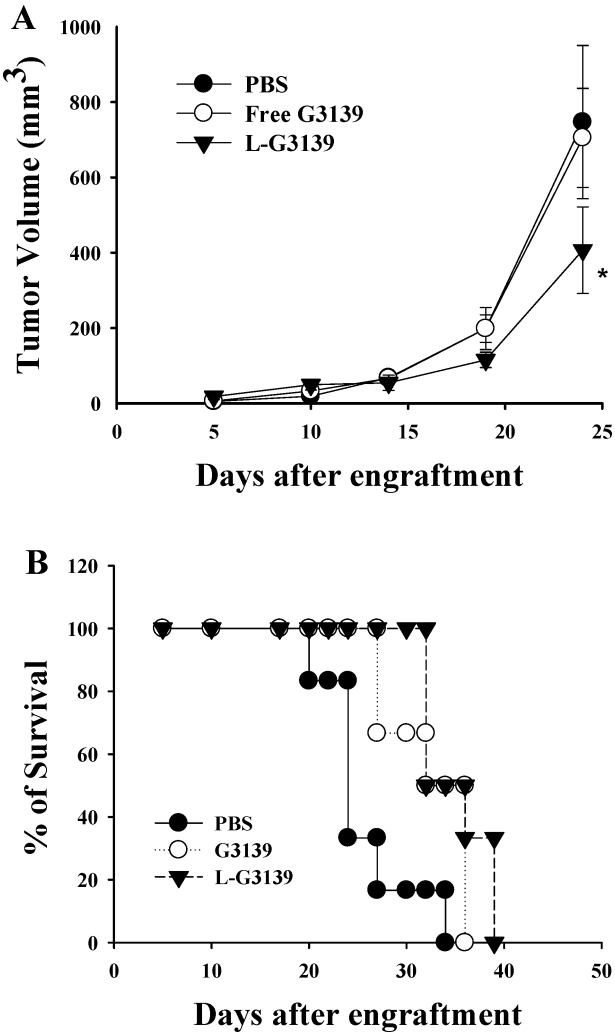

Figure 5. In vivo antitumor efficacy of G3139 in L1210JF bearing DBA/2 mice. Mice (6 per group) were implanted subcutaneously on day 0 with 1×10^6 L1210JF cells. On day 5, animals were treated with with 1 mg/kg of free G3139 or liposomal G3139. The treatment was continued every 4 days for four times. (A) Inhibition of tumor growth. Tumor dimensions were measured every 2 days using caliper. (B) Survival of L1210JF bearing mice after treatment with free G3139 or liposomal G3139. PBS was injected as a vehicle control. (*; $P < 0.05$ compared with PBS group.)

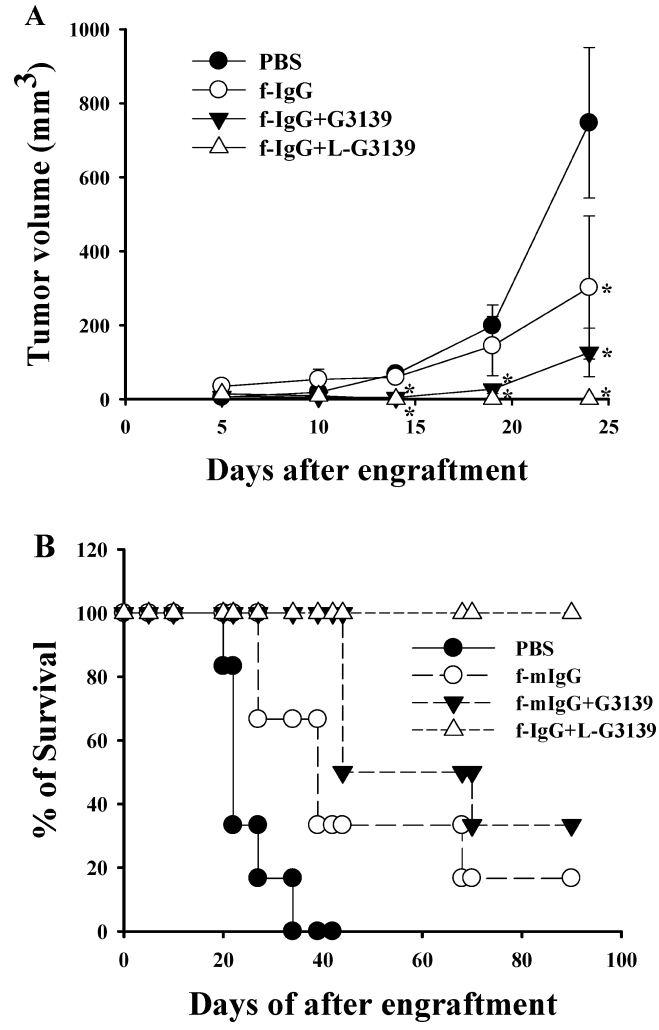

Figure 6. In vivo antitumor efficacy of f-mIgG combined with free G3139 or liposomal G3139 in L1210JF bearing DBA/2 mice. Mice (6 per group) were implanted subcutaneously on day 0 with 1×10^6 L1210JF cells. On day 5, animals were treated with 100 mg/kg of f-mIgG alone or combined with 1 mg/kg of free G3139 or liposomal G3139. The treatment was continued every 4 days for four times. (A) Inhibition of tumor growth. Tumor dimensions were measured every 2 days using caliper. (B) Survival of L1210JF bearing mice after treatment with free G3139 or liposomal G3139. PBS was injected as a vehicle control. (*; $P < 0.01$ compared with PBS group.)

that NK-mediated ADCC was FR-specific. Moreover, we demonstrated enhanced INF-γ production by NK cells when NK cells were co-cultured with f-IgG coated FR(+) tumor cells (*35*). Consistent with this, high levels of INF-γ and IL-6 were observed in the FR(+) tumor-bearing mice after administration of f-IgG compared with IgG (Figure 6), indicating that increased binding of f-IgG to tumor might elevate the level of cytokines. These results suggested that f-IgG might form a bispecific molecular bridge between the FR(+) tumor cells and immune effector cells that mediates tumor cell killing, through NK cell ADCC and cytokine production by NK cells, i.e., the same mechanism of a therapeutic antibody. A similar mechanism was reported by Lu et al. with a combination of a folate−hapten conjugate and endogenous antihapten antibody (*11, 12*). Therefore, the potent anticancer activity of f-IgG in FR(+) tumor-bearing mice observed was likely the result of FcR mediated ADCC and cytokine production, stimulated by f-IgG coated tumor cells via the bispecific molecular bridge. In addition, activated macrophages are known to express folate receptor (*36, 37*). Recently, Turk and Low showed that livers and spleens of tumor-bearing mice preferentially accumulated folate-targeted liposomes, presumably due to the FR-mediated targeting of agents to macrophages in livers and spleens (*38, 39*). Therefore, cytokine production by f-IgG accumulated macrophages in liver and spleen may also contribute to the anticancer activity of f-IgG in tumor-bearing mice. This mechanism remains to be investigated in the future studies.

G3139 was originally designed as a human bcl-2 mRNA antisense and has been investigated in phase I to III clinical trials as a monotherapy or combination with chemotherapy for treatment of chronic lymphocytic leukemia (CLL) (*40, 41*). Importantly, G3139 contains two CpG motifs, which have been shown to activate cytokine production via TLR9 (*25*). We therefore hypothesized that G3139 administration could enhance the antitumor effects of f-IgG. Free G3139 or liposomal G3139 have been shown to prolong animal survival. Compared to free G3139, liposomal G3139 was shown to be significantly more active in tumor inhibition (Figure 5). Recent studies in our lab have demonstrated that liposomal G3139 significantly increased plasma concentration of IFN-γ and IL-6 in mice, and increased proliferation of the DCs and NK cells (*27*). In contrast, no down-regulation of bcl-2 in murine L1210JF cells was observed, presumably due to sequence mismatches between G3139 and

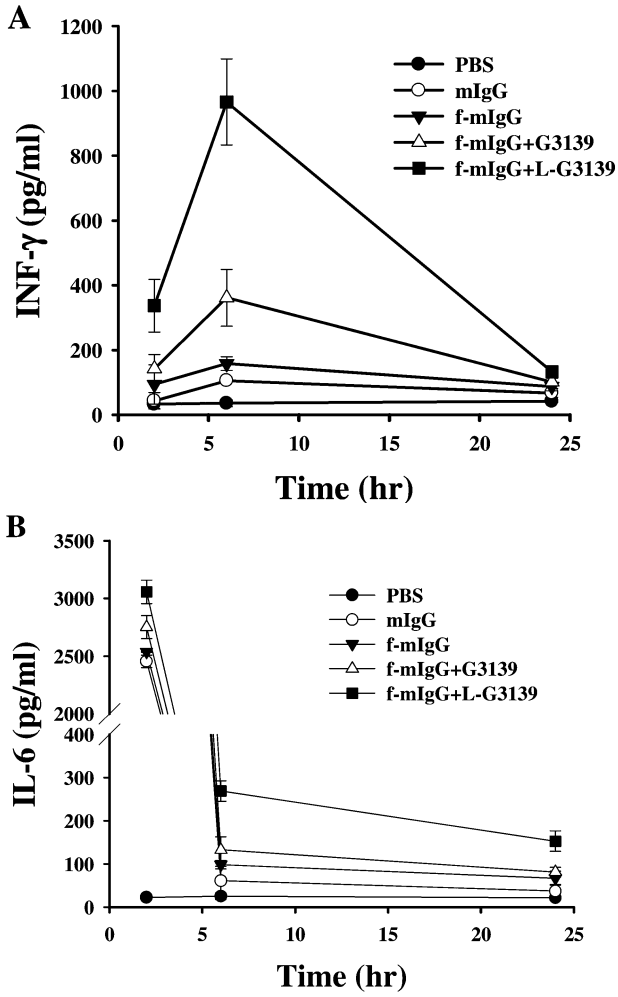

Figure 7. In vivo serum INF-γ (A) and IL-6 (B) production in L1210JF tumor-bearing mice. The tumor-bearing mice were treated with mIgG, f-mIgG alone or in combination with free or liposomal G3139. Blood samples were collected at 2, 6, and 24 h after injection. Serum was obtained by centrifugation and analyzed for INF-γ and IL-6 content by ELISA.

murine bcl-2 (*27*). These results suggested that the antitumor activity was not due to bcl-2 down-regulation but rather the immunomodulatory effect of G3139, which was amplified by liposomal delivery.

Moreover, a strong synergistic antitumor activity was observed when liposomal G3139 was used in combination with f-IgG (Figure 5). Consistent with this result, serum cytokine (INF-γ and IL-6) level was dramatically increased when f-IgG was combined with liposomal G3139 than combined with free G3139 (Figure 6). These results suggested that the synergic antitumor activity was due to the synergistically amplified cytokine (INF-α and IL-6) production in combination with liposomal G3139 and f-IgG. These results were consistent with previous reports (*11*), in which ADCC-activating cytokines such as interleukin 2 (IL-2) or interferon-alpha (IFN-α) have been shown to synergize with the folate receptor targeted immunotherapy.

CONCLUSIONS

F-IgG was shown to specifically target FR(+) tumor cells and be more effective against tumor compared to IgG in a murine tumor model. Moreover, the antitumor efficacy and the serum cytokine level were synergistically increased when co-administrated with liposomal G3139. A series of immunological assays indicated f-IgG's ability to mediate ADCC and cytokine production by immune cells. Therefore, f-IgG exhibits the characteristics of a therapeutic antibody. In addition, folate targeted f-IgG immunotherapy, combined with liposomal G3139, is a promising strategy for treatment of FR(+) cancers.

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