Preclinical pharmacokinetics, tissue distribution, and antitumor activity of a folate-hapten conjugate–targeted immunotherapy in hapten-immunized mice

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Abstract

Folic acid (pteroylglutamic acid) represents a useful ligand for targeted cancer therapies because it binds to a common epithelial tumor antigen known as the folate receptor. We previously devised an immunotherapy strategy that uses a bispecific ligand, a folate-hapten (FITC) conjugate, to redirect endogenously induced anti-FITC antibodies to folate receptor–positive tumor cells following parenteral administration. Here, we present results from preclinical pharmacokinetic and tissue biodistribution studies using a radioactive folate-FITC conjugate and results from dose optimization studies done in tumor-bearing animals. Folate-FITC was found to be rapidly eliminated in non-immunized mice; however, in immunized hosts, folate-FITC was shown to form immune complexes with FITC-specific antibodies, the consequence of which was a ~173-fold increase in drug exposure (i.e., area under the curve). Using a newly developed ELISA assay, the extent of circulating anti-FITC antibodies occupied by parenterally given folate-FITC was determined to be proportional to the given dose. Furthermore, high doses of folate-FITC were found to promote the cosaturation of tumor cell surface folate receptors and circulating FITC-specific antibodies, blocking the immune recognition of tumor cells and thereby reducing antitumor activity. Nonetheless, by extending the duration of treatment and administering subsaturating doses of folate-FITC, enhanced antitumor response was observed in mice bearing established folate receptor–positive M109 tumors. Overall, results from the present study may help to guide clinicians through on-going clinical investigations of folate-targeted immunotherapy. [Mol Cancer Ther 2006;5(12):3258–67]

Introduction

One of the objectives of a successful cancer immunotherapy is to ''teach'' the immune system to recognize and destroy malignant tumor cells and, in the process, build a long-lasting antitumor memory (1). The pronounced expression of the membrane-bound folate receptor among human neoplasms suggests that folate-targeted immunotherapy may be a promising therapeutic option (2–5). Previously, we developed an immunotherapy for cancer comprised of a vaccine, a folate-hapten conjugate, and immunostimulatory cytokine(s) (6–8). The vaccine component consisted of a hapten-protein conjugate formulated with a Th1-biased adjuvant to stimulate the production of anti-hapten antibodies. The hapten-vaccinated host later received the folate-hapten, which was thought to form a bispecific molecular bridge between tumor surface folate receptor and endogenous anti-hapten antibodies. This process effectively ''marks'' the tumor cells for immune recognition and initiates a specific antitumor response that could further be enhanced upon costimulation with interleukin-2 (IL-2) and IFN-α (6–8).

Based on experiments with mouse effector cells *in vitro*, folate-targeted immunotherapy was suggested to function via multiple tumor-killing mechanisms, including antibody-dependent cellular cytotoxicity and phagocytosis (8). Additional studies of immune effector cell involvement *in vivo* showed the participation of asialo-GM1$^+$ natural killer cells, macrophages, and CD4$^+$ and CD8$^+$ T cells in tumor regression (8). More importantly, a tumor-protective immunity was established in cured animals, and it was associated with the detection of memory CD4$^+$ and CD8$^+$ T cells *in vivo* and tumor-specific cytotoxic T-cell activity *ex vivo* (6, 8). Accordingly, the induction of humoral and cellular immune responses may have been complementary to each other in the process of both hapten-dependent and hapten-independent tumor cell destruction.

Preclinical work has thus far been focused on the therapeutic components that include (*a*) the fluorescein hapten (FITC), (*b*) keyhole limpet hemocyanin (KLH) as the carrier protein, (*c*) GPI-0100 as the adjuvant, (*d*) folate-ethylenediamine-FITC (folate-FITC) as the targeted hapten, and (*e*) the proinflammatory cytokines IL-2 and IFN-α (8). FITC was chosen as the hapten primarily because of its low molecular weight, ready availability, and ease of coupling

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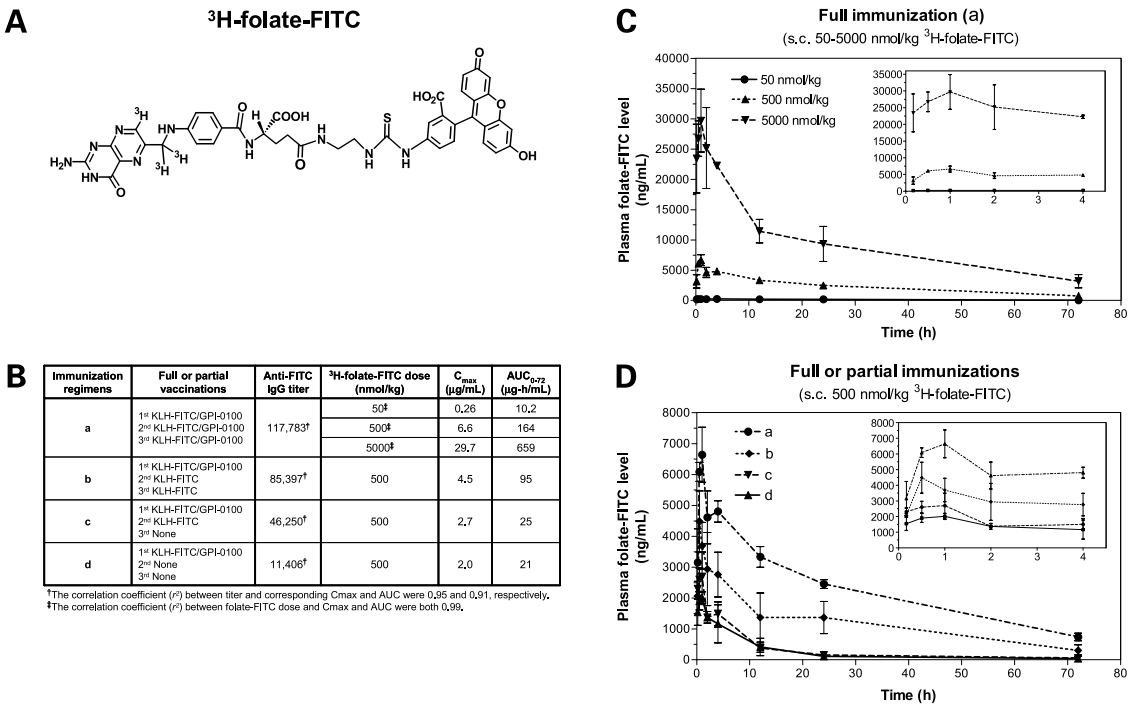
to KLH or folate (to make folate-FITC). FITC fluorescence also allowed for the optical examination of folate-FITC accumulation into folate receptor–positive tissues (9, 10). KLH was chosen as the carrier protein for FITC because of its well-known immunogenicity (11). KLH is also commonly employed as a carrier for cancer vaccine antigens, such as MUC1 peptide, mucin-like epitopes, and gangliosides (12–14). To enhance antibody production against KLH-FITC, GPI-0100, a Th1-biased saponin-based adjuvant (12, 14), was added to the vaccine formulation; importantly, high anti-FITC IgG titers (including IgG2a) resulted from this formulation. A variety of FITC conjugates of folic acid were prepared and tested preclinically; however, folate-ethylenediamine-FITC (molecular weight = 873) was selected for development because it exhibited good water solubility, rapid penetration into solid tumors, and high affinity for the folate receptor. Of the many cytokines available for testing, the combination of IL-2 and IFN-α (which are antibody-dependent cellular cytotoxicity–activating cytokines) was selected because it was found to produce a strong, synergistic activity in our preclinical models (6–8). The latter observation was important because it permitted the use of lower (and less toxic) cytokine dose levels.

Although encouraged by the above findings, little was known about the distribution properties of a folate-hapten conjugate within a hapten-immunized host. Therefore, one aim of this study was to investigate the pharmacokinetics and biodistribution of folate-FITC following administration to naive or FITC-immunized mice, either with or without a s.c. folate receptor–positive tumor. To aid our investigation, we developed a novel ELISA assay that measures the percentage of total serum-derived anti-FITC IgG antibodies that are occupied by folate-FITC. We examined various dosing combinations for folate-FITC, IL-2, and IFN-α in an effort to better define the therapeutic conditions in mice bearing disseminated peritoneal tumors and then re-examined the optimized therapy in animals bearing established s.c. tumors. Collectively, the results from this investigation may be useful for supporting ongoing immunotherapy clinical trials.

Materials and Methods

Reagents

Folate-(γ)-ethylenediamine-fluorescein (folate-FITC) and its radiolabeled form (^{3}H-folate-FITC) were synthesized at Endocyte, Inc., and the positions of tritium labels are depicted in Fig. 1A. KLH was purchased from Sigma Chemical Co. (St. Louis, MO). FITC (isomer I) was purchased from Molecular Probes (Eugene, OR). KLH-FITC conjugate was prepared as described previously (6). The saponin-based adjuvant GPI-0100 was obtained from Galenica (Birmingham, AL), and QS-21 was from



Immunization regimens	Full or partial vaccinations	Anti-FITC IgG titer	^{3}H-folate-FITC dose (nmol/kg)	C_{max} (μg/mL)	$AUC_{0\text{-}72}$ (μg-h/mL)
a	1st KLH-FITC/GPI-0100 2nd KLH-FITC/GPI-0100 3rd KLH-FITC/GPI-0100	117,783†	50‡	0.26	10.2
			500‡	6.6	164
			5000‡	29.7	659
b	1st KLH-FITC/GPI-0100 2nd KLH-FITC 3rd KLH-FITC	85,397†	500	4.5	95
c	1st KLH-FITC/GPI-0100 2nd KLH-FITC 3rd None	46,250†	500	2.7	25
d	1st KLH-FITC/GPI-0100 2nd None 3rd None	11,406†	500	2.0	21

†The correlation coefficient (r^2) between titer and corresponding Cmax and AUC were 0.95 and 0.91, respectively.
‡The correlation coefficient (r^2) between folate-FITC dose and Cmax and AUC were both 0.99.

Figure 1. Dose-dependent plasma clearance of ^{3}H-folate-FITC in mice fully or partially immunized against FITC. **A,** structure of ^{3}H-folate-FITC. **B,** table summarizing the various immunization regimens and their corresponding anti-FITC IgG titers, doses of ^{3}H-folate-FITC given, and values of pharmacokinetic analysis, including C_{max} and $AUC_{0\text{-}72\ h}$. **C,** pharmacokinetics of s.c. given ^{3}H-folate-FITC (50–5,000 nmol/kg) in mice given full vaccination. **D,** pharmacokinetics of s.c. given ^{3}H-folate-FITC (500 nmol/kg) in mice given full (*a*) or partial vaccinations (*b–d*). The plasma folate-FITC concentration (ng/mL) as a function of time was plotted over 4 h (*inset*) or 72 h after injection (**B** and **C**). *Points,* mean; *bars,* SD.

Antigenics (New York, NY). Human recombinant IL-2 was purchased from Peprotech (Rocky Hill, NJ) as a lyophilized powder with no additives. Human recombinant IFN-α A/D was purchased from PBL Biomedical Laboratories (Piscataway, NJ). For *in vivo* use, IL-2 and IFN-α were prepared in sterile PBS (pH 7.4) containing 1% syngeneic serum (6). Bovine milk folate-binding protein was purchased from Scripps Laboratories (San Diego, CA). Goat anti-mouse IgG biotin conjugate and streptavidin-horseradish peroxidase were purchased from Caltag Laboratories (Burlingame, CA). Folate-free rodent chow was obtained from Harlan TEKLAD (Madison, WI). All other reagents were purchased from major suppliers.

Animals, Cell Lines, and Tumor Models

Female BALB/c mice, 6 to 8 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and allowed to acclimate for at least 1 week before initiation of any experiment. Folate receptor–positive M109 tumor cells were cultured and regenerated as described previously (6). Only early-passage (P1) viable tumor cells were suspended in folate-deficient RPMI 1640 supplemented with 1% syngeneic mouse serum (antibiotic-free) and used for i.p. (5×10^5 per animal) or s.c. (1×10^6 per animal) tumor implantations. In general, mice were fed a folate-free diet for ~3 weeks before any folate-FITC or ^{3}H-folate-FITC administration to reduce serum folate levels to values characteristic of humans (~20 nmol/L; ref. 15).

Immunizations

Unless otherwise specified, we followed a standard protocol that generates consistently high titers in mice by immunizing thrice at 2-week intervals on days 0, 14, and 28 with 35 μg KLH-FITC admixed with 100 μg GPI-0100 (or 20 μg QS-21) as the adjuvant (this regimen represents a ''full'' vaccination, as noted herein). QS-21 is the natural adjuvant purified from the plant saponin, and its immunostimulatory property is similar to that of its semisynthetic analogue GPI-0100 (16, 17). For evaluating the effect of antibody titer on the pharmacokinetics of ^{3}H-folate-FITC, mice were given full or partial immunizations as described in Fig. 1B (i.e., adjuvants were omitted at the second or third vaccination, or vaccine injections were simply skipped). Serum samples from various studies were harvested as needed and analyzed for anti-FITC IgG titer following an established ELISA procedure (6). Antibody titer was defined as the serum dilution that yielded 50% of the maximal binding on a bovine serum albumin-FITC–coated plate.

Pharmacokinetics and Biodistribution Studies

Studies in this section included three mice per time point. Study I: Non–tumor-bearing mice that received full or partial vaccinations were given a single s.c. injection of ^{3}H-folate-FITC at 50, 500, or 5,000 nmol/kg on day 35. The animals were euthanized by CO_2 asphyxiation at designated time points (10 and 30 min, 1, 2, 4, 12, 24, and 72 h), and plasma samples were collected. Study II: Non–tumor-bearing naive or immunized mice were given a single s.c. or i.v. injection of ^{3}H-folate-FITC (500 nmol/kg) on days 35 or 53. Mice assigned to the latter date were pretreated with 14 doses of nonradioactive folate-FITC (s.c. 500 nmol/kg) on days 36 to 39, 42 to 46, and 49 to 52 (i.e., stopped 1 day before ^{3}H-folate-FITC administration). Plasma along with ~100 mg of tissues (heart, lung, spleen, liver, intestine, bile, feces, kidney, brain, and muscle) were collected for up to 168 h. Study III: Immunized mice bearing s.c. M109 tumors (~50 mm^3) were given a single injection of ^{3}H-folate-FITC (s.c. 1,800 nmol/kg) on day 38. Only plasma and folate receptor–positive tissues (tumor and kidney) were processed for radioactivity for a total of 120 h. All radioactive samples collected in this study were solubilized with Soluene-350 and decolored with 30% hydrogen peroxide. After adding 15 mL Hionic-Fluor fluid (Packard BioScience, Meriden, CT), the samples were light-adapted and counted for radioactivity in a scintillation counter. The sample-associated radioactivity was expressed as % injected dose (%ID) per gram, except for study I where folate-FITC concentration in the plasma was quantitated in terms of nanograms per milliliter.

Immunotherapy Studies

To better define the augmentative effect of cytokines, immunized mice (n = 4 per cohort) bearing 7-day-old i.p. M109 tumors were treated i.p. for 3 weeks with PBS or folate-FITC (1,800 nmol/kg, 5 days/wk) in combination with IL-2 (5,000–150,000 units/dose, 5 days/wk) and/or IFN-α (25,000–75,000 units/dose, 3 days/wk). The therapeutic outcomes were compared based on animal survival in each dose combination. To determine the efficacy against established solid tumors, immunized mice (n = 8 per cohort) with 10-day-old s.c. M109 tumors (~100 mm^3) were given 3 weeks of folate-FITC treatment (500–5,000 nmol/kg, s.c., five times per week) alone or in combination with IL-2 (20,000 units/d, five times per week) plus IFN-α (25,000 units/d, three times per week). The tumor dimensions were measured two to three times a week, and tumor volumes were calculated by the following formula: $V = 0.5ab^2$, where a is the longest axis across the tumor, and b is the shorter axis perpendicular to a. The mice were euthanized when the tumor volume reached ≥1,000 mm^3 and then counted for survival.

ELISA Analysis of Folate-FITC/Anti-FITC IgG Immune Complex in Mouse Serum

Bovine milk folate-binding protein–coated plates were preblocked with 0.2% gelatin in PBS containing 0.05% Tween 20. Serial dilutions of pooled mouse immune sera pre-added to various concentrations of folate-FITC (0–72 μmol/L) were added to the plates and incubated for 1 h at 37°C. After washing, the plates were incubated sequentially at 1-h intervals with a biotin-conjugated goat anti-mouse IgG secondary antibody (Caltag) and streptavidin-conjugated horseradish peroxidase (Caltag). The presence of folate-FITC/anti-FITC IgG immune complexes was revealed by adding *o*-phenylenediamine dihydrochloride in substrate buffer (Sigma Fast *o*-phenylenediamine dihydrochloride tablet sets). The average absorbance values at 490 nm were plotted against log serum dilution

factors (e.g., 1:100 dilution = $\log_{10}$ 100 = 2) to generate titration curves. EC_{50} is defined as the serum dilution that yields 50% of the maximal binding on the folate-binding protein–coated plate.

Determination of Percent Saturation of Anti-FITC IgG by Folate-FITC *Ex vivo*

Immunized mice (n = 3 per cohort) were dosed s.c. with folate-FITC at 500, 800, 2,000, or 5,000 nmol/kg. Mouse sera were collected 1 h after injection, pooled for each dose group, and stored frozen at −20°C. Before ELISA analysis, each serum sample was divided in half and then incubated with either PBS or folate-FITC (57 μmol/L) for 1 h at 37°C (serum dilution controlled to minimum). Equal volumes of the paired serum ± folate-FITC samples were added to a Bio-Spin P-30 column (Bio-Rad, Hercules, CA) and centrifuged to remove unbound folate-FITC. The presence of folate-FITC/antibody complex in both samples was detected by ELISA as described above. The percentage of anti-FITC IgG saturation by folate-FITC was calculated using the following formula: %IgG saturation = 100 × EC_{50} (serum + PBS) / EC_{50} (serum + folate-FITC).

Pharmacokinetic Analysis

Pharmacokinetic data analysis was done as needed using the WinNonlin software program (Pharsight Corp., Mountain View, CA). The following pharmacokinetic variables were obtained: area under the plasma concentration-time curve ($AUC_{0\text{-}t}$), maximum ^{3}H-folate-FITC concentration (C_{max}), time to reach C_{max} (T_{max}), total plasma clearance (CL and CL_F), distribution half-life [$t_{1/2}(\alpha)$], elimination half-life [$t_{1/2}(\beta)$], volume of distribution (V_z and V_z_F), and bioavailability (F). $AUC_{0\text{-}t}$ is the area from time 0 to the time (t) of last measurable concentration or last blood sample.

Statistics

Statistical analyses on survival curves were done using the log-rank test in the computer program GraphPad Prism (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant. Correlations among folate-FITC dose, antibody titer, and pharmacokinetic variables of folate-FITC, including C_{max} and $AUC_{0\text{-}t}$, were determined by simple linear regression analysis.

Results

Plasma Pharmacokinetics of Folate-FITC after Single or Repeat Administration

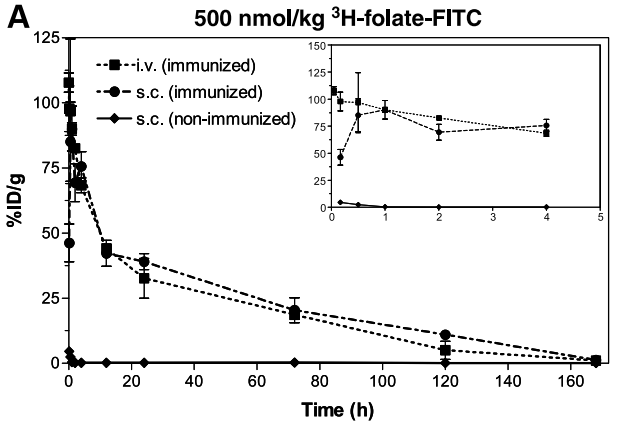
Because folate-FITC forms immune complexes in a FITC-immunized host, the rate of plasma disappearance of folate-FITC could conceivably be dependent on anti-FITC antibody titer/affinity and the amount of folate-FITC given. To test this hypothesis, mice that received full or partial vaccine regimens (to vary titer levels) were injected s.c. with 50 to 5,000 nmol/kg ^{3}H-labeled folate-FITC, and the plasma radioactivity was monitored for 72 h (Fig. 1). As shown in Fig. 1C and D (*inset*), s.c. given ^{3}H-folate-FITC appeared in the plasma within 10 min, peaked between 0.5 to 1 h, and then cleared more slowly in those mice that either (*a*) received a high folate-FITC dose (Fig. 1C), or (*b*) produced a higher anti-FITC antibody titer (Fig. 1D). The data analysis shown in Fig. 1B indicates a monotonic increase in C_{max} and $AUC_{0\text{-}72\ h}$ from 50 to 5,000 nmol/kg in mice with comparable titers (i.e., those that received a full vaccination regimen). Furthermore, when a fixed dose of folate-FITC (500 nmol/kg) was given to mice with variable titers, there was also a titer-dependent increase in C_{max} and $AUC_{0\text{-}72\ h}$.

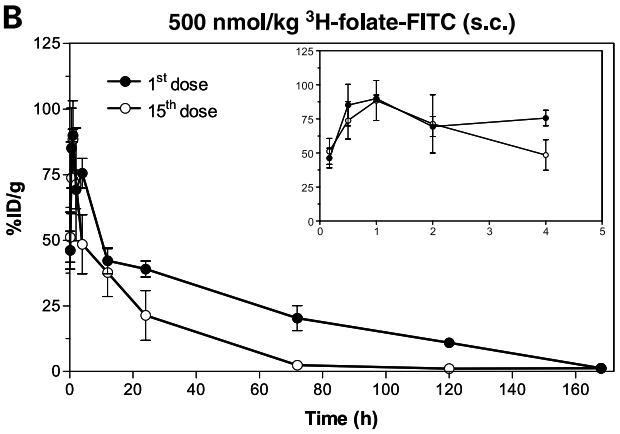
In a second study, fully immunized and non-immunized mice were given a single i.v. or s.c. injection of ^{3}H-folate-FITC (500 nmol/kg), and the plasma radioactivity was monitored for 168 h (Fig. 2A). As summarized in Fig. 2C, the calculated pharmacokinetic variables (e.g., $AUC_{0\text{-}168\ h}$, C_{max}, etc.) were found to be comparable between i.v. and s.c. administrations, suggesting that s.c. administration of folate-FITC provides for high bioavailability, a property also observed with other folate-targeted agents (18). Interestingly, in non-immunized mice, the majority of radioactivity was cleared from the plasma within 1 to 2 h, resulting in a very low $AUC_{0\text{-}168\ h}$ value of 1.8 μg h/mL (compared with i.v. at 267 μg h/mL, or s.c. at 312 μg h/mL for the immunized animals).

To investigate the effect of multiple dosing on the plasma clearance of folate-FITC, we predosed immunized mice with 500 nmol/kg of nonradioactive folate-FITC (a total of 14 daily doses) followed by a single injection of 500 nmol/kg ^{3}H-folate-FITC (i.e., 15th dose; Fig. 2B). As summarized in Fig. 2C, pretreatment with folate-FITC resulted in a significant reduction in the plasma clearance half-lives of ^{3}H-folate-FITC, which decreased from 36.5 h after the 1st i.v. dose to 12.2 h after the 15th i.v. dose. Independent of the administration route, there was also an approximate 2.5-fold reduction in $AUC_{0\text{-}168\ h}$ of ^{3}H-folate-FITC, which ranged from 267 μg h/mL (i.v.) or 312 μg h/mL (s.c.) after the 1st dose, to 113 μg h/mL (i.v.) or 125 μg h/mL (s.c.) after the 15th dose. The total plasma clearance rate (CL) also increased from 0.03 mL/h (1st dose) to 0.07 mL/h (15th dose). The apparent distribution volumes remained relatively unaffected over the same period, suggesting that the majority of folate-FITC was bound to plasma anti-FITC antibody after each dose.

Pharmacokinetics of Folate-FITC in Normal versus Tumor Tissues

Preliminary studies in naive mice and rats indicated that folate-FITC is eliminated from the body through both renal and hepatic routes (data not shown). Because the plasma clearance of folate-FITC was largely dependent on the host's immunization status (Fig. 1D), we investigated the pharmacokinetic biodistribution of ^{3}H-folate-FITC in both immunized and non-immunized mice (Fig. 3A and B). In non-immunized mice, ^{3}H-folate-FITC was rapidly eliminated via the hepatic route, with a significant amount of radioactivity detected in the bile/feces (32.30 ± 6.19, 319 ± 191, and 11.26 ± 9.31 %ID/g at 30 min, 4 h, and 24 h, respectively). Despite the high hepatic excretion rate, very little accumulation of the radioactivity was measurable in the liver or intestine over time and even less was found in other normal organs. Due to folate receptor expression on





C **Pharmacokinetic analysis**

Immunization Status		Immunized				Non-immunized[a]
Route of Administration		Intravenous Administration		Subcutaneous Administration		Subcutaneous Administration
		1st dose	15th dose	1st dose	15th dose	1st dose
Average Dose per Mouse (μg)		8.48 ± 0.55	8.62 ± 0.79	8.31 ± 0.59	9.08 ± 0.81	8.67 ± 0.70
Parameters	Units	Mean ± SE (n = 3)				
$AUC_{0\text{-}168}$	μg h/mL	267.1 ± 34.6	113.0 ± 17.0	311.7 ± 72.8	125.0 ± 31.6	~1.8
CL or CL_F	mL/h	0.03 ± 0.004	0.07 ± 0.01	0.024 ± 0.006	0.06 ± 0.02	ND
$T_{1/2}$ (α)	h	1.69 ± 0.43	0.362 ± 0.15	2.99 ± 2.33	0.40 ± 19.24	ND
$T_{1/2}$ (β)	h	36.54 ± 6.99	12.2 ± 2.5	48.80 ± 17.89	16.93 ± 7.07	ND
C_{max}	μg/mL	9.19 ± 0.24	11.70 ± 1.03	8.78 ± 13.93	7.94 ± 0.47	0.38 ± 0.02
V_z or V_z_F	mL	0.69 ± 0.14	0.52 ± 0.09	0.66 ± 0.28	1.06 ± 21.5	ND
F	%	-	-	130	104	-

[a] Since the plasma levels of folate-FITC in non-immunized mice were too low to be fitted into the two-compartmental model, only C_{max} and $AUC_{0\text{-}t}$ values were obtained using non-compartmental analysis. ND = a value that could not be accurately determined.

Figure 2. Comparison of route of administration and effect of multiple dosing on plasma clearance of ^{3}H-folate-FITC. **A,** fully immunized or non-immunized mice received a single i.v. or s.c. injection of ^{3}H-folate-FITC (500 nmol/kg). **B,** mice were immunized three times on days 1, 15, and 29. ^{3}H-folate-FITC (500 nmol/kg) was given either as the 1st dose on day 35, or as the 15th dose on day 53 following 14 pre-doses of non-radioactive folate-FITC (500 nmol/kg). Plasma-associated radioactivity in **A** and **B** was expressed as %ID/g and plotted over 4-h (*inset*) or 168-h observation period. *Points,* mean; *bars,* SD. **C,** summary table containing pharmacokinetic variables obtained by two-compartmental analysis.

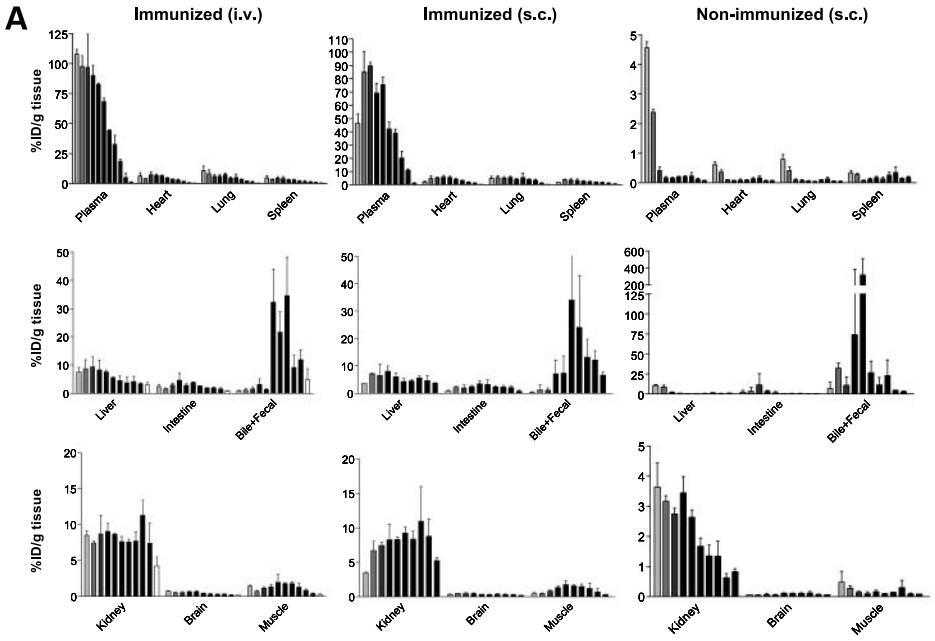
proximal tubule cells, kidney displayed higher uptake than other non-secretory folate receptor–negative tissues (e.g., heart, lung, spleen, and muscle). Although hepatic clearance was also apparent in immunized mice (e.g., 24.09 ± 18.86 ID/g in the bile/feces at 24 h after s.c. injection), the presence of anti-FITC antibody dramatically reduced the rate of ^{3}H-folate-FITC clearance and significantly increased its duration in the plasma and its retention in normal tissues. However, regardless of the route of administration (i.v. or s.c.), the overall tissue pharmacokinetic profiles of ^{3}H-folate-FITC were very similar after an initial phase of distribution in immunized mice (Fig. 3A and B).

Concerning the tumor uptake of folate-FITC, previous work with folate-based imaging agents of similar molecular sizes had indicated that a single bolus dose between 1,700 and 2,300 nmol/kg achieves folate receptor saturation *in vivo*, regardless of the level of folate receptors expression on a tumor (19). In one of our preliminary studies, we examined the M109 tumor uptake of ^{3}H-folate-FITC in non-immunized mice and found that tumor uptake increased 3-fold (4 h after administration) when the dose was increased from 500 to 2,000 nmol/kg (data not shown). Subsequently, immunized M109 tumor-bearing mice were given a single ''saturable'' s.c. dose of ^{3}H-folate-FITC (1,800 nmol/kg) to determine the kinetics of tumor uptake and to also compare that uptake with the radioactivity found in the kidney (a normal folate receptor–expressing organ) and plasma up to 120 h. As shown in Fig. 3C, a significant fraction (17.6% ID/g) of ^{3}H-folate-FITC was retained in the plasma within 10 min after injection, presumably binding to anti-FITC antibody; furthermore, the kidney captured 3.4% ID/g or ~92% of its maximal uptake capacity. In contrast, a slower and time-dependent accumulation of ^{3}H-folate-FITC was observed to occur in the folate receptor–positive tumor, where a plateau of ~ 4.2 to 4.5 %ID/g tissue was reached between 4 and 24 h after injection. At 120 h after injection, kidney and tumor retained >60% of their maximal accumulation, whereas only 2% of the plasma peak value was still detectable. These data reveal a significant and long-lasting accumulation of folate-FITC in folate receptor–positive tissues in the presence of anti-FITC antibody.

Effect of Folate-FITC Dosing on Percent Saturation of Anti-FITC IgG *In vivo*

Although pharmacokinetic studies of ^{3}H-folate-FITC have provided evidence of immune complex formation in immunized mice, the amount of radioactivity found in the plasma was not solely due to antibody binding. In other words, folate-FITC can also bind nonspecifically to serum proteins (mostly serum albumin) at a level of ~74% (i.e., high-performance liquid chromatography analysis of a 30-kDa microfiltrate following incubation at 50 μmol/L ^{3}H-folate-FITC with human serum). Thus, an ELISA assay that employs immobilized folate-binding protein was developed to directly assess the formation of folate-FITC/anti-FITC IgG complexes. Here, bovine milk folate-binding protein is adsorbed to high protein-binding polystyrene plates and used to capture folate-FITC/antibody complexes. Briefly, by incubating mouse immune sera with or without excess folate-FITC (with subsequent removal of unbound folate-FITC by microcentrifugation), the differences in signals from matched sample pairs were calculated,

and the values were used to calculate the percentage of anti-FITC IgG saturation (refer to Materials and Methods). As shown in the assay development in Fig. 4A and B, there was a linear relationship between the %IgG saturation (with folate-FITC) and the amount of folate-FITC added to the immune sera until saturation (arbitrarily defined as 100%) was reached at ~18 μmol/L. Notably, the mouse immune sera used for this experiment were collected from mice 1 week after receiving a 3rd KLH-FITC vaccination (boosters occurring at 2-week intervals), which was approximately the time that we routinely began folate-FITC treatment in our therapy studies. To assess the %IgG saturation *in vivo*, immunized mice were given a single s.c. dose of folate-FITC ranging from 500 to 5,000 nmol/kg,



B

Percentage of Injected ^{3}H-Folate-FITC Dose per Gram of Tissue Following a Single Administration*

Immunization Status	Non-immunized		Immunized			
Route of Administration	Subcutaneous		Subcutaneous		Intravenous	
Time of Collection	4 h	24 h	4 h	24 h	4 h	24 h
Plasma	0.16 ± 0.04	0.21 ± 0.04	75.59 ± 5.62	39.00 ± 3.05	68.31 ± 2.78	32.61 ± 7.68
Heart	0.10 ± 0.03	0.14 ± 0.03	5.47 ± 1.06	3.28 ± 0.24	4.72 ± 0.13	2.90 ± 0.81
Lungs	0.06 ± 0.02	0.10 ± 0.01	4.14 ± 0.99	3.97 ± 0.55	4.44 ± 1.14	3.24 ± 0.65
Spleen	0.18 ± 0.04	0.27 ± 0.10	2.56 ± 0.58	1.92 ± 0.36	2.78 ± 0.90	1.64 ± 0.75
Liver	0.40 ± 0.11	0.67 ± 0.15	6.10 ± 1.24	4.53 ± 0.50	5.50 ± 0.30	3.65 ± 2.02
Intestine	1.59 ± 1.09	0.60 ± 0.18	3.50 ± 1.19	2.37 ± 0.37	3.79 ± 0.31	1.91 ± 0.14
Bile+Feces	318.5 ± 191.4	11.26 ± 9.31	7.41 ± 6.26	24.09 ± 18.86	32.26 ± 11.78	34.65 ± 13.60
Kidneys	2.63 ± 0.25	1.35 ± 0.36	8.31 ± 0.38	8.37 ± 1.17	7.60 ± 0.73	7.73 ± 1.25
Brain	0.11 ± 0.02	0.11 ± 0.01	0.36 ± 0.02	0.38 ± 0.09	0.46 ± 0.03	0.31 ± 0.06
Muscle	0.16 ± 0.06	0.14 ± 0.02	1.77 ± 0.56	1.49 ± 0.34	1.78 ± 0.12	1.28 ± 0.56

*Results are tabulated as mean ± s.d. of data (n = 3).

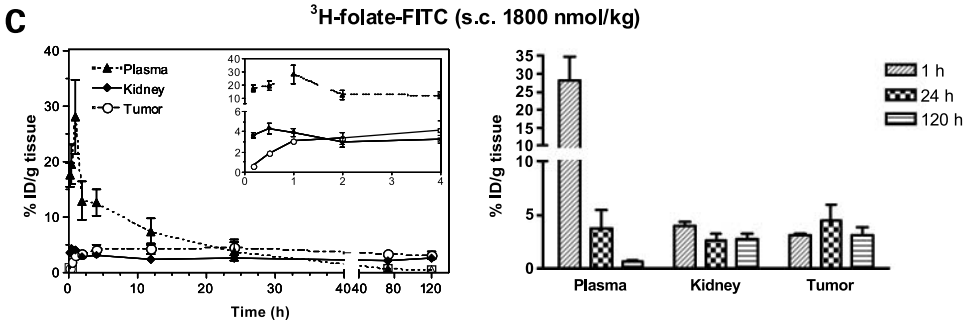


Figure 3. Pharmacokinetic biodistribution of ^{3}H-folate-FITC in FITC-immunized and non-immunized mice following systemic administration. **A,** mice were given ^{3}H-folate-FITC (500 nmol/kg) via a single i.v. or s.c injection. The time points, as shown from the left to the right for each tissue set in each of the nine bar graphs, were collected 3 (i.v. only), 10, and 30 min, 1, 2, 4, 12, 24, 72, 120, and 168 h. **B,** summary table containing the above values of ^{3}H-folate-FITC tissue distribution at 4 and 24 h after injection. **C,** comparison of ^{3}H-folate-FITC pharmacokinetics (s.c., 1,800 nmol/kg) in plasma, kidney, and tumor in immunized mice bearing s.c. M109 tumors (~50 mm^{3}). Plasma or tissue-associated radioactivity was expressed as %ID/g. *Points/columns,* mean; *bars,* SD.

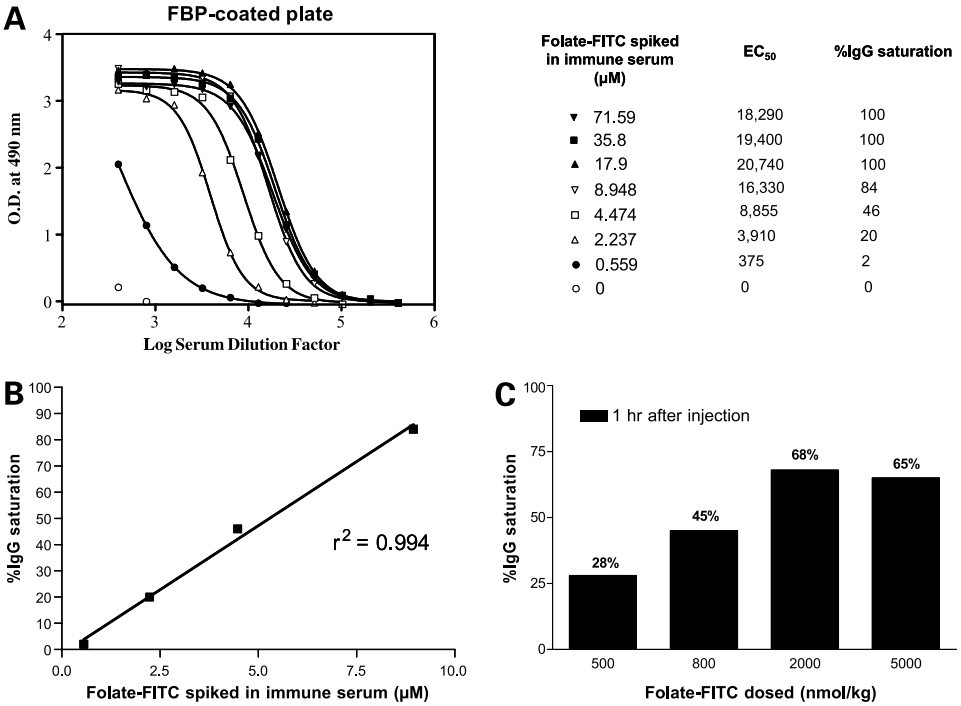


Figure 4. Percent saturation of anti-FITC IgG antibody by folate-FITC in immunized mice. **A,** ELISA titration curves of immune complexes in FITC-antiserum added to various concentrations of folate-FITC. **B,** linear regression analysis for %IgG saturation (anti-FITC) and serum folate-FITC concentration (used data from **A**). **C,** %IgG saturation in immunized mice injected with folate-FITC. See Materials and Methods for experimental details. O.D., absorbance.

and serum samples were collected at 1 h after injection (i.e., near C_{max}). As shown in Fig. 4C, a dose-dependent percent saturation of total anti-FITC IgG antibody was observed until a plateau of 65% to 68% was reached at the 2,000 to 5,000 nmol/kg dose levels. This result suggested that a 2,000 nmol/kg dose of folate-FITC allowed for maximal saturation of serum-derived antibody *in vivo*. Interestingly, this dose level is also known to allow for the saturation of tumor-derived folate receptors (see above).

Therapeutic Dosing Effect of Cytokines at a Fixed Dose of Folate-FITC

Besides tumor targeting, folate-FITC serves a key role in recruiting anti-FITC antibodies to folate-FITC ''marked'' tumor cells. Consequently, folate-FITC can promote antibody-dependent cellular cytotoxicity, phagocytosis, and perhaps T cell–mediated tumor cell killing (8). In the tumor destruction process, however, costimulation with the proinflammatory cytokines IL-2 and IFN-α seemed to be important in promoting a more effective and long-lasting antitumor response (8). Because high cytokine doses are often associated with unwanted toxicity, we investigated the effect of using lower doses of IL-2 and IFN-α in support of a fixed dosing regimen of folate-FITC. As shown in Fig. 5, immunized mice bearing 7-day-old i.p. M109 tumors were given a 3-week regimen consisting of folate-FITC (1,800 nmol/kg, five times per week) plus various dose levels of IL-2 (five times per week) and IFN-α (three times per week), alone or in combination. For all but one cytokine dose combination (i.e., 150,000 units IL-2 and 25,000 units IFN-α), an improved cure rate was observed in the folate-FITC plus cytokine(s) cohorts compared with the cytokine(s) alone. Overall, the preferred cytokine dose combination (for mice) consisted of <40,000 units IL-2 and 25,000 units IFN-α. At these levels, 100% cures were routinely achievable with slight to no observable gross toxicity.

Therapeutic Dosing Effect of Folate-FITC at a Fixed Cytokine Regimen

It has been shown that folate-FITC can efficiently target folate receptor–positive tumors in both primary and metastatic sites (10). Tumor accumulation of folate-FITC is folate receptor specific because it can be effectively blocked by 100-fold molar excess of free folic acid *in vivo* (10). The results of our pharmacokinetic studies in immunized mice (Fig. 3C) revealed that although systemically given ^{3}H-folate-FITC could localize quickly to a folate receptor–positive tumor, the majority of the compound was simultaneously bound to anti-FITC antibody in the plasma. We have previously observed that folate-FITC present in vast excess over anti-FITC antibody may actually avert the ''anchoring'' of anti-FITC antibody to folate receptor–positive tumor (7, 8). In an effort to consolidate our findings and to further enhance antitumor activity, immunized mice bearing s.c. M109 tumors (~100 mm^3) were given a 3-week regimen (dosing five times per week) consisting of folate-FITC at three dose levels (500, 2,000, and 5,000 nmol/kg), alone or in combination with IL-2 and IFN-α. The cytokine doses consisted of 20,000 units IL-2 at five-times-a-week schedule and 25,000 units IFN-α following a three-times-a-week schedule. The therapeutic end points included growth inhibition, survival (cutoff at ~1,000 mm^3 tumor volume), and overall cure rates after

a sufficient amount of time had passed to ensure no relapse of tumor.

As shown in Fig. 6A, administration of folate-FITC therapy without cytokines yielded 37.5% tumor-free mice (3 of 8) in the 500 nmol/kg dose and 12.5% (1 of 8) in both the 2,000 and 5,000 nmol/kg dose groups. The antitumor effect of folate-FITC alone was only statistically significant at the 500 nmol/kg dose when compared with that of the PBS control (**, $P = 0.003$). For the folate-FITC plus cytokine cohorts, there were 87.5% to 100% tumor-free mice in the 500 to 2,000 nmol/kg dose groups and 62.5% (5 of 8) in the 5,000 nmol/kg dose group (Fig. 6B). Notably, 25% (2 of 8) of the mice in the cytokine only treated cohorts were tumor-free, and this activity was statically significant compared with that of PBS (*, $P = 0.04$). Interestingly, no i.p. tumor-bearing animal was cured when treated i.p. for 3 weeks with 50,000 units IL-2 and 25,000 units IFN-α (see Fig. 5). However, such difference in the observed antitumor effect of cytokines was likely due to the differences in tumor location and the route of administration used for these two independent studies. Overall, the differences in antitumor activity between cytokines alone and cytokines plus folate-FITC at the 500 or 2,000 nmol/kg dose levels were statistically significant (**, $P = 0.0025$), but the antitumor effect of folate-FITC at 5,000 nmol/kg plus IL-2/IFN-α was not statistically different from that of cytokine treatment alone. In both cases (± cytokines), a reduction of therapeutic efficacy was observed for animals treated with the large 5,000 nmol/kg folate-FITC dose level. These results collectively suggest that the optimal therapeutic dose for folate-FITC (when given five times per week for 3 weeks) in immunized mice may be around 500 nmol/kg.

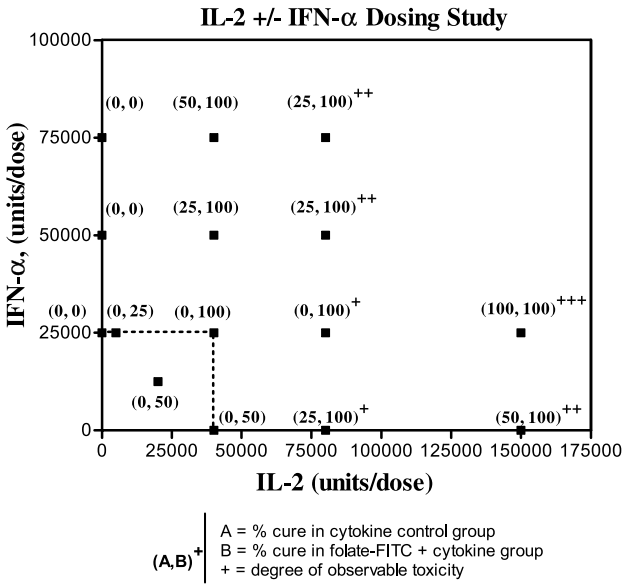


Figure 5. Effect of cytokine dose and dose combination on folate-FITC immunotherapy against peritoneal M109 tumors. KLH-FITC/QS-21–immunized mice ($n = 4$ per group) were implanted intraperitoneally on day 0 with 5×10^5 viable tumor cells. Various doses of IL-2 (five times per week) and IFN-α (three times per week) alone or in combination with folate-FITC (1,800 nmol/kg, five times per week) were given i.p. on days 7 to 11, 14 to 18, and 21 to 25 after tumor cell implantation. Gross toxicity at the time of treatment was scored based on the following visual assessment: +, slightly rough far coat; ++, rough fur coat, slightly swollen abdomen; +++, very rough fur coat, swollen abdomen, and/or diarrhea, etc.

Discussion

We have examined the pharmacokinetic biodistribution of a folate-hapten conjugate in both hapten-immunized and non-immunized hosts in an effort to better understand the factors that mediate efficacy for the treatment of folate receptor–positive tumors. Using FITC as the model hapten, a water-soluble folate-FITC conjugate was constructed and found to modestly bind serum proteins but not erythrocytes. The radiolabeled form of folate-FITC was found to eliminate quickly in non-immunized mice through both renal and hepatic routes, but the rate of plasma clearance was largely reduced in the presence of endogenous anti-FITC antibody (i.e., in vaccinated animals). The longer plasma retention of folate-FITC in immunized hosts may be viewed as an advantage due to its potential relocation to folate receptor–positive tumors. Overall, the pharmacokinetic profile of folate-FITC was found to be directly dependent on dose and anti-FITC antibody titer (see Fig. 1).

Because administration of excess folate-FITC should saturate both anti-FITC antibodies and tumor surface folate receptors with separate folate-FITC molecules, thereby preventing the ''docking'' of anti-FITC antibodies to the tumor cells, we developed an ELISA method capable of measuring the percent saturation of anti-FITC IgG antibody in sera collected from immunized mice. Following a standard immunization regimen, this technique helped us to confirm that the plasma-derived, FITC-specific IgG molecules in mice that received a ≥2,000 nmol/kg dose of folate-FITC were occupied to nearly 70% (see Fig. 4). The fact that 100% antibody saturation was not achieved *in vivo* was puzzling. However, it may reflect the dynamics of active immunization, extravasation of folate-FITC, the presence of other FITC-specific antibody isotypes (IgM and IgE), and/or the limitation of such ''snap-shot'' *ex vivo* sampling. Nevertheless, these data provided evidence that the excessive folate-FITC doses can negatively influence therapeutic outcome (see below).

Earlier studies have shown that coadministration of IL-2 and IFN-α with folate-FITC produces remarkable life extension (including cures) in mice bearing i.p. tumors (6–8). By extending the duration of treatment and optimizing the dose levels of the individual therapeutic components, a new regimen consisting of folate-FITC (500–2,000 nmol/kg) plus IL-2 (20,000 units) and IFN-α (25,000 units) was found to produce superior antitumor activities, including in some cases of up to 100% cures in mice originally bearing well-established s.c. tumors (see Fig. 6). However, consistent with what had previously been observed in animal tests (8), as well as what was predicted

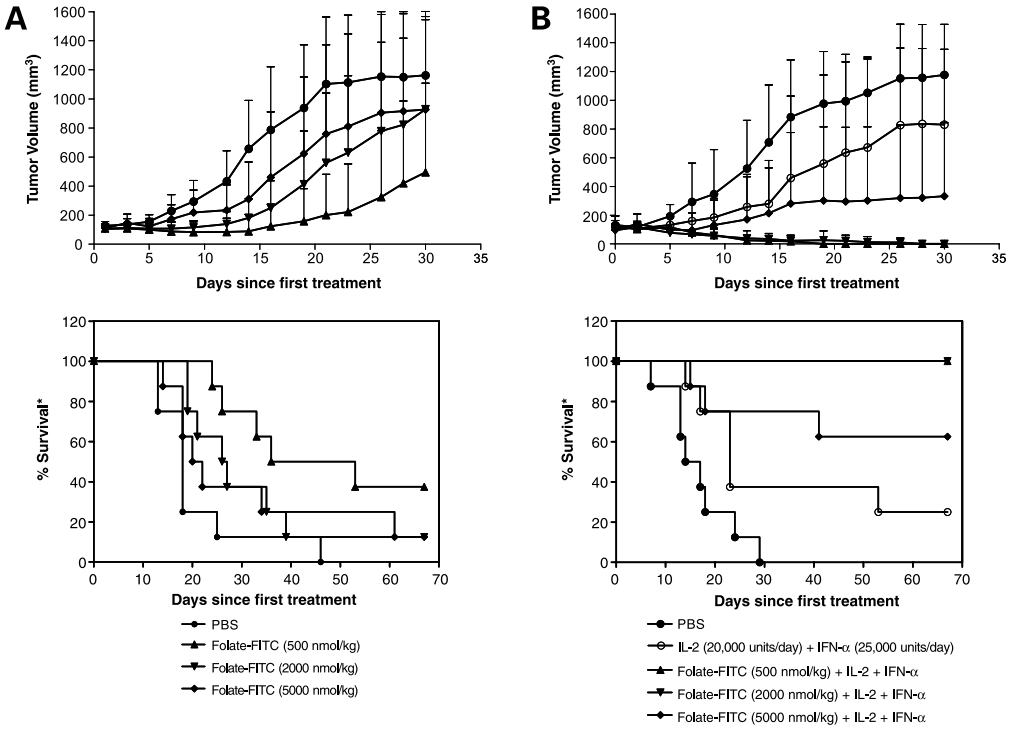


Figure 6. Effect of folate-FITC alone or in combination with cytokines against established s.c. M109 tumor. KLH-FITC/GPI-0100–immunized mice (n = 8 per cohort) were implanted s.c. on day 0 with 1×10^6 viable tumor cells. Treatments were given on days 10 to 14, 17 to 21, and 24 to 28. Tumor volumes and % survival were monitored as described in Materials and Methods. *Points,* mean of eight animals; *bars,* 0.5 SD (above point only). **A,** folate-FITC was given alone at 500 (▲), 2,000 (▼), or 5,000 (♦) nmol/kg (five times per week). **, P = 0.003 when the group treated with 500 nmol/kg folate-FITC is compared with the PBS group. **B,** IL-2 (20,000 units, five times per week) and IFN-α (25,000 units, three times per week) were given in the absence (○) or presence of 500 (▲), 2,000 (▼), or 5,000 (♦) nmol/kg folate-FITC (five times per week). *, P = 0.04 when the cytokine treatment group is compared with the PBS group. **, P = 0.0025 when the folate-FITC (500–2,000 nmol/kg) plus cytokine treatment groups are compared with the group treated with cytokines alone. Note, tumor in one of the eight mice within the 2,000 nmol/kg folate-FITC plus cytokine group (▼) had recurred 81 d following initial treatment; this event dropped the overall survival rate down to 87.5% (data not shown).

from the aforementioned ELISA assay, less favorable results were obtained in animals given 5,000 nmol/kg folate-FITC for the 3-week duration. Generally, it would seem that multiple injections of a folate-hapten dose (e.g., 500 nmol/kg) that is well below saturation of the host's anti-FITC antibodies should be optimal.

With multiple dosing, one might not expect to see a loss in targeting efficiency because the binding affinity of our folate-hapten conjugate for tumor cell folate receptor is in the nanomolar range, which is probably higher than the average affinity of polyclonal antibodies generated against the hapten (20, 21). In our FITC-immunized mice, we did observe an increase in plasma clearance and a decrease in $AUC_{0\text{-}168\ h}$ after repeated administration of folate-FITC (see Fig. 2). The reasons for such altered pharmacokinetics could be due to (*a*) the saturation of available binding sites in the body by folate-FITC, (*b*) the removal of folate-FITC/IgG immune complexes by the reticuloendothelial system, and/or (*c*) the high metabolic rate of rodents.

Admittedly, when compared with single-agent therapies (e.g., monoclonal antibodies), the folate-hapten–targeted immunotherapy is a more complicated process requiring vaccination before drug treatment. Nevertheless, it may have several advantages over conventional cancer vaccine strategies that derive from (*a*) the high tumor specificity of folate conjugates, (*b*) the rapid and noninvasive conversion of a non-immunogenic tumor to an immunogenic tumor by the folate-hapten conjugate, and (*c*) the potential induction of both antibody-dependent (humoral) and antibody-independent (cellular) antitumor responses. Compared with the targeting of a cytotoxic agent that requires intracellular delivery of the free drug (protein toxins, chemotherapeutics, etc.), this approach of folate receptor–targeted immunotherapy is not subjected to the limitation of folate receptor–mediated endocytosis. The folate-hapten conjugates are likely to be more stable and perhaps less toxic *in vivo* compared with conjugates of cytotoxic agents. In addition, tumor resistance and recurrence are common with the use of chemotherapeutic drugs, which typically requires multiple mechanisms of action to achieve a long-lasting antitumor response. When successful, a cancer immunotherapy would ''teach'' the immune system to recognize/destroy malignant tumor cells and, in the process, build a long-lasting memory against tumor antigens to prevent the recurrence of the same or related tumor cells.

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