Research Article

Theme: Critical Variables in the In Vitro and In Vivo Performance of Parenteral Sustained Release Products
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Strategy to Prevent Drug-Related Hypersensitivity in Folate-Targeted Hapten Immunotherapy of Cancer

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Abstract. Cancer vaccine/immunotherapy rarely involves systemic administration of an immunogenic compound to an actively immunized host. We have developed such a strategy that utilizes folate to deliver antigenic haptens [e.g., fluorescein (FITC) and dinitrophenyl] to folate receptor-positive tumors in a hapten-pre-vaccinated host. Here, we investigated the safety of this novel approach and developed strategies to prevent drug-related hypersensitivity. Using FITC as the model hapten, we identified a potential source of allergic species in folate-FITC preparations by LC-MS/MS. In mice and guinea pigs, we tested the significance of this impurity by passive cutaneous anaphylaxis and active systemic anaphylaxis assays. We studied the effect of immunogen (e.g., KLH-FITC) dose and derived a desensitization regimen that was further evaluated in a murine tumor model. Administration of folate-FITC with low multi-haptenated contaminants (e.g. bis-FITC) resulted in hypersensitivity in under-immunized animals. However, this drug-related hypersensitivity may be independently prevented by (1) increasing the immunogen dose and/or (2) desensitizing animals with folate-FITC during vaccination. In addition, such manipulation *in vivo* did not appear to negatively alter the effectiveness of immunotherapy. This study provided confidence on the safety of folate-hapten-targeted cancer immunotherapy in an actively immunized host.

KEY WORDS: cancer immunotherapy; folate targeting; hapten; hypersensitivity.

INTRODUCTION

The folate receptor (FR), identified in human cancers through immunohistochemical analyses (1) and real-time imaging with folate-linked radiopharmaceuticals (2–4), constitutes a potential therapeutic target. Although the exact function is unclear, epithelial cancer cells tend to overexpress FR- α , which can bind folate–drug conjugates with nanomolar affinity and bring a fraction of the surface-bound molecules inside the cell via endocytosis (5). As therapeutic monoclonal antibodies have gained increasing popularity due to human-

ization, high specificity, and low toxicity (6), we developed an alternative strategy of targeting endogenous antibodies to FR-positive cancer via a low-molecular-weight folate–hapten conjugate, namely, folate-targeted hapten immunotherapy (7–9). Here, folate–hapten conjugates serve as a bispecific molecular “bridge” between tumor cells and the endogenous anti-hapten antibody induced by vaccination. This folate-targeted opsonization process initiates antitumor immune responses that include antibody-dependent cellular cytotoxicity, phagocytosis, and subsequent development of cellular immunity (10).

Although no allergic reaction to any folate-conjugated haptens [e.g., fluorescein (FITC) and dinitrophenyl (DNP)] was ever observed during therapy in preclinical modeling, we have reported that folate–DNP conjugates exhibited a greater risk of allergy if they shared an identical hapten linker chemistry to that found in its keyhole limpet hemocyanin (KLH)-based immunogen (8). Still, the allergic potential of folate–DNP conjugates was only detectable in a sensitive rat passive cutaneous anaphylaxis (PCA) assay. Using FITC as the model hapten and KLH–FITC as the immunogen, the present study was designed to investigate the risk factors associated with the administration of a folate–FITC conjugate in KLH–FITC-immunized mice and guinea pigs. In order to

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ABBREVIATIONS: ASA, Active systemic anaphylaxis; BSA, Bovine serum albumin; DNP, Dinitrophenyl; EDA, Ethylenediamine; FITC, Fluorescein; FR, Folate receptor; HPLC, High-performance liquid chromatography; IFN- α , Interferon- α ; IL-2, Interleukin-2; KLH, Keyhole limpet hemocyanin; LC-MS/MS, Liquid chromatography–mass spectrometry/mass spectrometry; PCA, Passive cutaneous anaphylaxis.

induce active systemic anaphylaxis (ASA) in these animals, we reduced the KLH-FITC vaccination dose and then challenged them with a folate-FITC product that contained small amounts of bis-FITC, a highly allergic bis-haptenated impurity that was identified during the folate-FITC manufacturing process. Once this model was in place, we then devised “early desensitization” strategies to circumvent the observed allergic reaction and also determined whether such manipulations had any adverse effects on immunotherapy against an FR-positive murine tumor. The conclusions from this investigation, in terms of safety and efficacy, may provide guidance towards the clinical investigation of folate-FITC-targeted immunotherapy.

MATERIALS AND METHODS

Materials

The structures of folate-FITC and bis-FITC are shown in Fig. 1. Folate-FITC (i.e. folate- γ -ethylenediamine-FITC) was synthesized as described previously with modifications (7). Briefly, folate- γ -ethylenediamine (11) was coupled to FITC isomer I (Sigma-Aldrich, St. Louis, MO) in anhydrous dimethylsulfoxide in the presence of tetramethylguanidine and diisopropylamine. The crude product was loaded onto an Xterra RP18 preparative HPLC column (Waters) and eluted with gradient conditions starting with 99% 5 mM sodium phosphate (mobile phase A, pH7.4) and 1% acetonitrile (mobile phase B) and reaching 90% A and 10% B in 10 min at a flow rate of 20 mL/min. Under these conditions, the folate-FITC main peak typically eluted at 27–50 min, whereas bis-FITC remained on the column. The quality of each folate-FITC fraction was monitored by analytical reverse-phase HPLC with a UV detector. Fractions with greater than 98.0% purity and undetectable bis-FITC (LC-

MS) were lyophilized to obtain the final folate-FITC product. The absence of bis-FITC in the final product was further confirmed by LC-MS/MS. bis-FITC was also made separately by reacting FITC with ethylenediamine followed by HPLC purification. Re-EC20 is a rhenium-labeled peptide derivative of folic acid analogous to ^{99m}Tc -EC20 (12). It was prepared by published methods (13,14).

KLH-FITC conjugate was manufactured at CIS-US, Inc. (Bedford, MA) with a labeling ratio of ≥ 120 μmol of FITC per gram of KLH. GPI-0100, a semi-synthetic plant saponin adjuvant, was obtained from Galenica (Birmingham, AL). Bovine serum albumin (BSA)-FITC was synthesized as described previously (7). A biotinylated BSA-FITC was made by reacting BSA-FITC with EZ-LinkTM sulfo-NHS-LC-biotin following manufacturer's instructions (Pierce). Human recombinant interleukin (IL)-2 was purchased as a lyophilized powder from PeproTech (Rocky Hill, NJ). Human recombinant interferon (IFN)- α A/D was purchased from PBL Biomedical Laboratories (Piscataway, NJ). For *in vivo* use, IL-2 plus IFN- α were prepared in sterile phosphate-buffered saline (PBS; 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH7.4) containing 1% syngeneic mouse serum and stored at -80°C in small aliquots. All other biologic or chemical reagents were purchased from commercial sources.

Animals and Diets

Female Balb/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN), acclimated after 1 week and used when they reached 6–8 weeks of age. To generate allergy test serum, Balb/c mice were immunized (s.c.) against either 1 or 35 μg of KLH-FITC plus 100 μg of GPI-0100 at 1-week intervals. FITC-antiserum samples were collected 2 weeks after the last immunization. Female Lewis rats

Fig. 1. Structures and purity profiles of folate-FITC and bis-FITC. Shown are structures of folate-FITC and bis-FITC, a bis-haptenated impurity formed during folate-FITC synthesis and removed by repeat HPLC purifications

(~3.5 month of age, 200–224 g) were purchased from Harlan and used for PCA assay (see below). For anaphylaxis assays, all animals (mice, rats, and guinea pigs) were fed regular rodent diets. For tumor study, mice were fed a folate-deficient diet (Harlan Teklad, Indianapolis, IN) for ~3 weeks prior to the first dose of folate-FITC. This is a standard practice to lower plasma folate levels to avoid competition for the FR-targeted folate-FITC (9). All animal studies were done in accordance with AAALAC guidelines.

ELISA Analysis

The level of anti-FITC IgE in mouse serum was measured by a capture enzyme-linked immunosorbent assay. Briefly, plates coated with anti-mouse IgE capture mAb (BD Biosciences) were blocked with PBS containing 1% BSA for 30 min. After washing, serial dilutions of pooled FITC antiserum was added to the plates and incubated for 1 h. The plates were washed and incubated further with biotin-BSA-FITC as the primary reagent and streptavidin-conjugated horseradish peroxidase as the secondary reagent. Finally, the presence of mouse anti-FITC IgE antibodies was revealed by adding *o*-phenylenediamine dihydrochloride in substrate buffer (Sigma Fast™ *o*-phenylenediamine dihydrochloride tablet sets). The enzymatic reaction was stopped by the addition of 3 N HCl, and the optical density was read at 490 nm.

Passive Cutaneous Anaphylaxis Assay

The source of “allergic” FITC antiserum used for the PCA assay was obtained from mice immunized against 35 µg KLH-FITC plus GPI-0100 (see above). Briefly, Lewis rats were sensitized intradermally (i.d.) on the shaved dorsal surface with 0.1 mL of serial dilutions (1:3, 1:6, 1:12, 1:24, and 1:48) of FITC antiserum pooled from the donor mice. The injection spots were circled with a marker. Forty-eight hours later, the animals were challenged intravenously (i.v.) with 6.7 µg of bis-FITC, 1 mg BSA-FITC (multi-haptenated positive control), and equal molar amount of fluoresceinamine (51 µg, mono-haptenated control) and folate-FITC (134 µg) in 1% Evans Blue dye solution. Thirty minutes after challenge, the rats were euthanized by CO₂ asphyxiation, and the areas of blue spots were measured.

Active Systemic Anaphylaxis Assay

The ASA assay schemas used for testing folate-FITC hypersensitivity in mice and guinea pigs are illustrated in Fig. 2. In mice, animals were first immunized on days 1, 8, and 15 with 1 or 35 µg KLH-FITC plus 100 µg GPI-0100. They were challenged i.v. on day 22 with PBS or various preparations of folate-FITC at either 500 or 1,500 nmol/kg (Fig. 2a). The body temperature of each mouse was measured using a rectal probe designed specifically for mice (RET-3, Thermocouple Thermometer). The baseline temperature was taken immediately prior to injection up to ~30 min post-challenge (and as frequently in-between as necessary). Animals were euthanized by CO₂ when they displayed severe shock with no activity after prodding and/or when their body temperature had dropped by ≥3°C or more. To test the effect

Fig. 2. ASA vaccination schema. The schemas shown were used for the active systemic anaphylaxis (ASA) assay to assess folate-FITC hypersensitivity in KLH-FITC/GPI-0100 immunized mice and guinea pigs. **a, b** Animals were vaccinated on days 1, 8, and 15 (V) and challenged with a test article (TA) on day 22. **b** Desensitization was done by daily dosing on days 8–12 and 15–19

of FR blocking on folate-FITC hypersensitivity, a separate group of mice were injected s.c. with a 60-fold molar excess of Re-EC20 (12) at 1–2 min and 4 h before a folate-FITC challenge. For desensitization studies (Fig. 2b), mice were given daily s.c. doses of a folate-FITC product either with or without 10 mol% of bis-FITC on Days 8–12 and 15–19. These animals were then subjected to an i.v. challenge on day 22 with the same dose of the corresponding folate-FITC +/-bis-FITC formulation.

For guinea pigs, male and female animals (one per sex per group) were immunized on days 1, 8, and 15 with 13.3, 33, 100, and 200 µg of KLH-FITC formulated with 0.5 mg of GPI-0100. On day 22, the animals were challenged s.c. with a single dose of folate-FITC (1.27 mg/kg), bis-FITC (1.07 mg/kg), or folate-FITC (1.14 mg/kg) plus 10 mol% bis-FITC (0.107 mg/kg). For desensitization purposes, a separate group of guinea pigs were given multiple s.c. doses of folate-FITC containing 10 mol% bis-FITC on days 8–12 and 15–19. On day 22, these animals were again challenged with the correspondingly same folate-FITC/bis-FITC formulations. Clinical observations were periodically taken up to 2 h post-challenge. Animals were euthanized if they displayed signs of a severe anaphylactic shock. Complete macroscopic postmortem examinations were performed on all animals.

Tumor Study

The murine 4T1c2 tumor cell line is derived from 4T1 mammary carcinoma stably transfected with the murine FR gene (9). The cells were maintained in folate-free RPMI 1640 medium (Gibco BRL) supplemented with 10% v/v heat-inactivated fetal calf serum. A total of 2.5×10^5 of the 4T1c2 cells were used to generate subcutaneous (s.c.) tumors in syngeneic female Balb/c mice. For therapy studies, mice were immunized s.c. for three times at 2-week intervals with 35 µg

of KLH-FITC plus 100 μ g of GPI-0100. The animals were implanted s.c. with 250,000 viable 4T1c2 tumor cells according to two different schedules shown in Fig. 3. In the first schedule (Fig. 3a), treatment with folate-FITC plus IL-2/IFN- α started 13 days after the third immunization. In the second schedule with desensitization (Fig. 3b), folate-FITC treatment started early on the day of second immunization (day 8), and IL-2/IFN- α were added 3 days after the third immunization. For both treatment regimens, folate-FITC (500 nmol/kg) and IL-2 (20,000 U) were dosed at five times/week, while IFN- α (25,000 U) was dosed at 3 times/week. The tumor dimensions were measured two to three times a week, and tumor volumes were calculated by the following formula: $V = 1/2 \times a \times b^2$, where a is the longest axis across the tumor and b is the shorter axis perpendicular to a .

Statistical Analysis

Statistical analysis on tumor growth curves was performed using the computer program GraphPad Prism (GraphPad Software Inc., San Diego, CA). Differences in tumor growth were considered significant when $P \leq 0.05$.

RESULTS

Identification of bis-FITC, a Multi-haptenated Impurity in a Folate-FITC Product

Folate-FITC is generally prepared by reacting folate- γ -ethylenediamine with FITC (isomer I) in the presence of

tetramethylguanidine and diisopropylamine. Folate- γ -ethylenediamine was initially prepared by reacting folic acid- γ -methyl ester with excess ethylenediamine. Unreacted ethylenediamine was removed by passing through diethylamino ethanol ion-exchange column; trace amounts of the ethylenediamine linker remain and subsequently react with FITC to form a bis-haptenated side product, bis-FITC-EDA (bis-FITC; see Fig. 1). Although a majority of the bis-FITC impurity can be removed by preparative HPLC purification (see Materials and methods), additional HPLC purifications are necessary to reduce the impurity to undetectable levels in each folate-FITC product. Also shown in Fig. 1 are typical HPLC profiles of a folate-FITC product before and after removal of bis-FITC by additional HPLC methods. Unless otherwise noted in the text, all folate-FITC products used in the present study were deemed to be free of bis-FITC, as confirmed by LC-MS/MS (data not shown).

Bis-FITC was Highly Allergenic in Rats Pre-sensitized with Mouse FITC Antiserum

Previously, we have immunized mice against 35–50 μ g KLH-FITC plus various adjuvants (TiterMax Gold®, Alum, GPI-0100, QS-21, etc.), and no symptoms of allergy to any folate-FITC product was ever observed (Table I). Since IgE is a good marker for allergy and its production is an indication of suboptimal immunogen dose, we first assessed FITC-specific IgE antibody in mice immunized against 1 or 35 μ g of KLH-FITC plus 100 μ g GPI-0100 (see “**MATERIALS AND METHODS**”). As shown in Fig. 4a, despite the fact that GPI-0100 is considered a Th1-biased adjuvant (15), mice immunized with KLH-FITC/GPI-0100 were observed to generate anti-FITC IgE at levels that were inversely dependent on the KLH-FITC dose used during the vaccination process. This suggested that an allergic IgE response was possible in immunized animals treated with hapten-based formulations containing bis-FITC (see classical mechanism in Fig. 5). However, to better assess the allergic potential of the folate-FITC and bis-FITC products, we employed a standard PCA protocol (16,17) in rats that had been intradermally pre-sensitized with an “allergic” FITC antiserum. Rats were given 2 days to “washout” any anti-FITC IgG from the injection site to favor an IgE-specific response. Each test article was given as an i.v. challenge to the IgE-sensitized rats in the presence of Evan’s Blue dye. Consequently, any allergic reaction at the sites of antiserum inoculation could be visualized by cutaneous extravasation of the dye. As shown in Fig. 4b, there was a significant reduction in hypersensitivity between the initial folate-FITC product and the HPLC repurified (i.e., non-detectable bis-FITC-containing) product ($P < 0.05$). Importantly, BSA-FITC and fluoresceinamine, the respective multi-haptenated and mono-haptenated controls, produced their predicted positive and negative responses. Under the same conditions, bis-FITC, at a dose as low as 6.7 μ g per rat, was found to produce the strongest cutaneous reaction, thereby confirming its ability to cause serious allergy in a FITC-immunized host. Unfortunately, these data showed that repurification of the folate-FITC product largely reduced but did not completely eliminate its allergic potential.

Fig. 3. Therapeutic schema. The schemas shown are schedules that were used to compare antitumor activity of folate-FITC immunotherapy **a** without or **b** with early folate-FITC desensitization in an FR-positive murine 4T1c2 tumor model (see Fig. 8)

Table 1. Lack of Folate-FITC Hypersensitivity in Animals Previously Immunized Against KLH-FITC plus Various Adjuvants

Species	KLH-FITC dose (μ g)	Adjuvants tested	References
Mouse	35–50	TiterMax Gold GPI-0100 (100 μ g) QS-21 (20 μ g) Alum	(10,24,39)
Rat	150	TiterMax Gold Alum	(38)
Guinea pig	200 2,000	GPI-0100 (500 μ g)	GLP study ^a
Monkey	600	GPI-0100 (1 mg)	Non-GLP study ^a

KLH-FITC keyhole limpet hemocyanin–fluorescein, GLP good laboratory practice

^a Unpublished results, Endocyte Inc.

Low Immunogen Dose Increased Folate-FITC Hypersensitivity but Such Effect Was Not FR-Mediated

While the PCA assay is “analogous” to the skin test used to predict drug allergies in humans, it would be difficult to correlate IgE levels in the rat skin to a systemic IgE allergy in an immunized host. For this reason, we sought to investigate the systemic nature of folate-FITC hypersensitivity using the ASA assay (see “MATERIALS AND METHODS”). Referring back to Fig. 2, this assay was done by i.v. challenge of mice that had been immunized against KLH-FITC (1 μ g versus 35 μ g) with a folate-FITC preparation declared to be free (i.e., undetectable by LC-MS/MS) of bis-FITC. Symptoms of hypersensitivity were monitored immediately after i.v. injection and included itching/scratching, redness, labored breathing, and decreased core body temperature measured by a rectal probe. As shown in Fig. 6a, mice that had been given the “optimal” 35 μ g KLH-FITC dose showed no signs of allergy and no changes in body temperature after receiving a challenge with 1,500 nmol/kg of the repurified folate-FITC product. However, mice that received the suboptimal 1 μ g KLH-FITC dose had experienced an allergic reaction and decreased body temperature shortly after receiving the same folate-FITC challenge. The observed hypersensitivity was also dose dependent, with less body temperature drop measured in animals dosed with 500 nmol/kg of folate-FITC compared to 1,500 nmol/kg (Fig. 6b).

Since FRs have been found on activated monocytes (18) and IgE receptors are expressed on basophils, we contemplated the possibility that the folate-FITC allergic response could be caused by the cross-linking of basophils and monocytes while in the circulation (see alternative mechanism in Fig. 5). To address this issue, a group of mice were injected with a 60-fold excess of Re-EC20 (a competitor for FR binding) prior to receiving an intravenous challenge with the folate-FITC product. As shown in Fig. 6c, blocking of folate-FITC binding to FR with Re-EC20 did not improve but rather appeared to have worsened the observed allergic response. From these observations, it is tempting to consider how excess Re-EC20 could have blocked FR-specific and non-specific binding sites in the body leaving more of the

folate-FITC product available to react with mast cells and/or basophils. However, additional studies are needed to support this hypothesis.

Folate-FITC Hypersensitivity in Mice was Prevented by Desensitization During Vaccination with the Same Folate-FITC Preparation

As shown above, folate-FITC hypersensitivity may be prevented by increasing the amount of KLH-FITC immunogen dose. However, in situations where the mice were given a suboptimal low KLH-FITC dose, more FITC-specific IgE was produced, and the animals experienced a hyper-

Fig. 4. **a** Anti-FITC IgE levels in mice and **b** folate-FITC hypersensitivity in a rat PCA assay. **a** Balb/c mice were immunized against 1 or 35 μ g KLH-FITC plus GPI-0100 on days 1, 8, and 15. The relative levels of anti-FITC IgE on day 29 were measured by a capture ELISA assay. **b** Lewis rats were injected intradermally in duplicates with serial dilutions of a mouse FITC antiserum (see “MATERIALS AND METHODS”). Forty-eight hours later, the rats were challenged i.v. with bis-FITC (6.7 μ g), BSA-FITC (1 mg), fluoresceinamine (51 μ g), or folate-FITC preparations (134 μ g) in 1% Evans Blue dye solution. Thirty minutes post-challenge, the rats were euthanized, and the areas of blue spots were measured and averaged between two spots per rat and two rats per test article. Each bar set, as shown from the left to the right for each test article, represents areas measured at 1:3, 1:6, 1:12, 1:24, and 1:48 serum dilutions. * $P < 0.05$ when the initial folate-FITC product was compared to the re-purified folate-FITC product

Fig. 5. Proposed mechanisms of folate-FITC hypersensitivity in an actively immunized host. Illustrated on the left are classical type I anaphylaxis caused by IgE sensitization of mast cells (and basophils) and subsequent exposure to a multivalent allergen (i.e., bis-FITC). Illustrated on the right is an alternative allergic situation that may occur during cross-linking of basophils and FR-positive monocytes in the circulation

sensitivity reaction toward folate-FITC despite the extensive repurification to remove trace amounts of the bis-FITC contaminant (Fig. 6). In order to ensure the safety of folate-FITC at all times, a desensitization strategy was evaluated by initiating folate-FITC dosing during the immunization process (i.e., starting day 8; see Fig. 2b). Mice treated in this way were then i.v. challenged with a defined and extensively repurified folate-FITC product on day 22 to assess its anaphylactic potential. As shown in Fig. 7a, early dosing with the highly purified folate-FITC product was found to eliminate the risk of allergy in mice that had been vaccinated with a suboptimal level of immunogen. Importantly, early dosing also prevented an allergic response in animals that received a folate-FITC product, which had been “artificially spiked” with 10 mol% bis-FITC, a response that was independent of the immunogen dose (compare Fig. 7b and c).

Increasing Immunogen Dose and Dosing Folate-FITC Early Alleviated Hypersensitivity in Guinea Pigs

To support an Investigational New Drug Application, we conducted an extensive pre-clinical good laboratory practice (GLP) toxicity study in ~400 guinea pigs and had observed no allergic responses to folate-FITC when guinea pigs were immunized against 200–2,000 µg KLH-FITC formulated with 0.5 mg of GPI-0100 (Table II). Since we had successfully “created” allergy in mice by decreasing the KLH-FITC dose and/or challenging immunized mice with bis-FITC spiked into the folate-FITC product (vide supra), we decided to evaluate the same impact of KLH-FITC dose reduction on hypersensitive/anaphylactic reactions induced by folate-FITC administration in a guinea pig ASA model (Table II). Cohorts

of two animals each (one male/one female) were immunized with different amounts of KLH-FITC (13.3, 33, 100, and 200 µg), while the amount of GPI-0100 was held constant. The animals were immunized once weekly for 3 weeks (days 1, 8, and 15) and then challenged on day 22 with a formulation of folate-FITC ± 10% bis-FITC (total folate-FITC concentration of 1.2 µmol/kg). Given the sensitive nature of guinea pigs, body temperature was not measured, but the animals were monitored for symptoms of allergic reaction, such as scratching, nose rubbing, piloerection, weakness, dyspnea, coughing, sneezing, or wheezing. After being observed for at least three time points up to 2 h post s.c. folate-FITC challenge, the animals were euthanized and necropsied. As shown in Table II, folate-FITC alone at 1.27 mg/kg caused only minor allergic reaction with no abnormality at the time of necropsy; in contrast, bis-FITC at 1.07 mg/kg caused convulsion, cyanosis, and even death in animals immunized against 13.3 µg of KLH-FITC (i.e., the lowest dose tested). Signs of cyanosis were also evident upon necropsy in these animals with darkened stomach, cecum, liver, and brain. However, as the KLH-FITC dose was increased from 13.3 to 100–200 µg, the hypersensitive symptoms decreased and organ abnormality diminished, even when the animals were challenged with 1.143 mg/kg folate-FITC plus 0.107 mg/kg bis-FITC. Thus, increasing the KLH-FITC amount in the vaccine formulation was found to decrease organ pathology in response to the challenge with a hypersensitivity-inducing test formulation (i.e., 10% bis-FITC spiked into the folate-FITC product). More importantly, early desensitization with 10% bis-FITC-spiked folate-FITC completely eliminated the hypersensitivity response in guinea pigs that had been immunized against the low KLH-FITC dose (13.3 µg).

Early Dosing of Folate-FITC Did Not Adversely Impact Antitumor Activity

While dosing folate-FITC early during immunization served the purpose of eliminating any allergic response in immunized animals regardless of immunogen dose, the

Fig. 6. Effect of immunogen dose on folate-FITC hypersensitivity. Folate-FITC hypersensitivity was assessed in mice following the ASA protocol, as described in Fig. 2a. **a** Mice immunized with 1 or 35 µg of KLH-FITC were i.v. challenged with 1,500 nmol/kg folate-FITC; **b** mice immunized with 1 µg of KLH-FITC were challenged with 500 or 1,500 nmol/kg folate-FITC; **c** mice immunized with 1 µg of KLH-FITC were challenged with 1,500 nmol/kg folate-FITC in the presence or absence of 60-fold molar excess of Re-EC20. The lines in each group ($n=3$) represents rectal temperatures recorded immediately before and after i.v. challenge. Control mice ($n=2$) were challenged with PBS only. **c** One mouse was challenged with Re-EC20 as a control

impact that this maneuver had on antitumor activity was called into question. We have previously shown that the administration of folate-FITC supplemented with low to moderate doses of IL-2±IFN- α can generate cures or significantly extend the lifespan of FITC-immunized, M109 tumor-bearing mice (7,8). The M109 tumor is a weakly immunogenic FR-positive subclone of the Madison lung carcinoma cell line that requires regular passages in Balb/c mice (19). In order to have a tumor that would continue to grow during the desensitization phase, we decided to test the concept of early folate-FITC dosing in a more resistant syngeneic 4T1c2 tumor model (9). 4T1c2 is a poorly immunogenic, highly metastatic breast cancer cell line stably transfected with murine FR- α at a level comparable to M109 tumor cells (~100 pmol/mg cellular protein, unpublished results, Endocyte Inc.). For comparison of efficacy with and without early folate-FITC desensitization, two different experiment schedules and dosing regimens were employed in an effort to match tumor sizes as closely as possible at the time of cytokine addition (Fig. 3). Previously, we have shown that vaccination alone did not have any antitumor effect (9). Briefly, mice were immunized, implanted s.c. with 4T1c2 tumors, and dosed with folate-FITC (500 nmol/kg, five times/week) supplemented with IL-2 (20,000 U, five times/week) and IFN- α (25,000 U, three times/week) as described in "MATERIALS AND METHODS." In the first regimen without desensitization (Fig. 3a), a 3-week treatment of folate-FITC plus IL-2/IFN- α was started 13 days after the third immunization with an average tumor size of ~110 mm³ (19 days post-implantation). In the second regimen (Fig. 3b), dosing with folate-FITC alone was started 1 week after the first immunization for desensitization purposes. When the tumor reached ~70 mm³ beginning 3 days after the third immunization (27 days post-implantation), IL-2/IFN- α were added along with folate-FITC for an additional 3 weeks of treatment.

Although it was not possible to match tumor sizes and antibody titers exactly at the time of cytokine addition, treatment of folate-FITC plus cytokines in the presence or absence of early desensitization had similar effects against established 4T1c2 tumors ($P>0.05$, Fig. 8). Notably, the tumor growth rates in control animals were similar in both regimens (regardless of times of tumor inoculation in relative to vaccination), and cytokines alone had minimal effect on tumors of similar sizes ($P>0.05$, compared to its control). While no cures were observed in the 4T1c2 tumor model, there was a statistically significant delay in tumor growth after the folate-FITC/cytokine therapy in both regimens (** $P<$

Fig. 7. Desensitization effect. **a** Dosing folate-FITC early prevented its hypersensitivity in KLH-FITC-immunized mice treated with normal or **b, c** a highly allergic folate-FITC product that was artificially spiked with bis-FITC. The dosing regimens for this ASA assay are shown in Fig. 2a, b (early desensitization). **a, c** Mice immunized with 1 μg or **b** 35 μg KLH-FITC were challenged with 1,500 nmol/kg folate-FITC spiked **b, c** with or **a** without 350 nmol/kg of bis-FITC (a potent allergen). The lines in each group ($n=3$) represents rectal temperatures recorded immediately before and after i.v. challenge. Control mice ($n=2$) were challenged with PBS only

0.01, compared to their corresponding controls). Finally, no allergic reaction or gross toxicity (weight loss/rough fur coat, etc.) was observed throughout the entire study, suggesting that this form of therapy is well tolerated.

DISCUSSION

One risk for administering a folate-hapten conjugate to a hapten-immunized host is the potential for the host to develop hypersensitivity to the folate-hapten conjugate. Prior to this report, no hypersensitivity to any folate-targeted haptens (FITC and DNP) had been observed in preclinical GLP and non-GLP studies involving mice, rats, guinea pigs, and monkeys (Table I). In a previous publication, DNP-immunized mice were found to produce anti-DNP IgE antibodies, and folate-DNP conjugates with identical hapten linker chemistry to that found in KLH-DNP (i.e. the immunogen) were found to cause allergy in a rat PCA assay (8). As most allergic and hypersensitivity reactions are associated with Th2-biased immune responses, the formation of allergen-specific IgE antibody is a hallmark of allergic sensitization (20,21). The fact that none of the KLH-hapten-immunized, folate-hapten-treated animals ever displayed any symptoms of allergy was puzzling, but this effect may depend on the extent to which the immune system is biased toward a Th2 response. In a published study (22), atopic human subjects were immunized intranasally with various doses of KLH (0.1, 10, 1,000, and 100,000 μg of KLH) in combination with a pro-allergic adjuvant. As the KLH dose increased, there was a gradual decline in KLH-specific IgE production and an increase in FITC-specific IgG production in nasal lavage samples. More importantly, the allergic sensitization rates for subjects in the 10-, 1,000-, and 100,000- μg -dose groups decreased from 100% to 57% and ultimately to 11%, respectively.

To identify the allergenic component in our study, we first examined the synthetic process of folate-FITC and identified a bis-haptenated impurity (i.e., bis-FITC). This impurity was formed to a small extent during the synthesis of folate-FITC as a result of the bifunctional ethylenediamine linker, and it was found to be highly allergenic in PCA rats pre-sensitized with mouse FITC antiserum (Fig. 4b). Next, we successfully induced a folate-FITC allergy in KLH-FITC-immunized mice and guinea pigs by lowering the immunogen dose during vaccination. Under these conditions, an immediate allergic reaction was produced, and the animals experienced an abrupt drop in body temperature (mice, Fig. 6) or cyanosis (guinea pigs, Table II) after being challenged with a 10% bis-FITC spiked folate-FITC product. These data suggested that folate-FITC allergy has the characteristics of

Table II. Folate-FITC Hypersensitivity in Guinea Pigs Evaluated by Active Systemic Anaphylaxis

Dosing schemas	KLH-FITC ($\mu\text{g}/\text{animal}$)	Test article formulation		Clinical observations		
		Folate-FITC (mg/kg)	Bis-FITC-eda (mg/kg)	M	F	Necropsy observations
(Fig. 2a)	13.3	0	0	–	–	No abnormalities reported
	13.3	1.27	0	$\pm$	$\pm$	No abnormalities reported
	13.3	0	1.07	++	++	M/F: Cyanosis (darkened stomach, cecum, liver and brain)
	13.3	1.14	0.107	+++	+	M/F: Stomach - dark serosa/lumen/mucosa;
	33	1.14	0.107	+	+	F: Liver-pale foci
	100	1.14	0.107	$\pm$	$\pm$	F: Stomach- dark serosa/lumen/mucosa
	200	1.14	0.107	+	+	M: Liver-pale foci
				$\pm$	$\pm$	No abnormalities reported
(Fig. 2b)	13.3	1.14	0.107 (10 mol%)	+	+	No abnormalities reported
				–	–	No abnormalities reported

Clinical evaluation keys: (–) no signs, ($\pm$) scratching/nose rubbing, and/or piloerection, (+) weakness, dyspnea, coughing, sneezing, and/or wheezing, (++) convulsions, cyanosis, and/or collapse, (+++) death
 KLH-FITC keyhole limpet hemocyanin–fluorescein

a type I hypersensitivity reaction mediated by mast cells and/or basophils. Evidence further supporting such a mechanism derives from (1) the observed production of FITC-specific IgE antibody and (2) the positive outcomes of the rat PCA assay (Fig. 4). We then speculated that once host mast cells or basophils became sensitized by anti-FITC IgE; subsequent exposure to any bis-FITC or bis-FITC-like species (in folate-FITC preparations) could crosslink the IgE/IgE receptor on these cells and cause a rapid degranulation (i.e., release of histamine, platelet-activating factor, and other vasodilators; Fig. 5, illustration on the left). As reported in the literature, allergen-specific IgG antibody can also cause type II or III hypersensitivity reactions due to its cytotoxic action on drug-hapten-coated blood cells (e.g., penicillin-induced hemolytic anemia) or the formation of drug-antibody complexes (e.g., serum sickness), respectively (23). Although folate-hapten conjugates can bind activated FR-positive monocytes in the bloodstream, folate-hapten-induced type II hypersensitivity was not deemed likely for the following reasons: (1) none of the aforementioned animal species experienced anemia following any folate-hapten therapy and (2) blocking of the FR with Re-EC20, another folate ligand, during folate-FITC challenge failed to reduce the hypersensitivity response. For the same reason, the probability that the folate-hapten conjugate promotes monocytes/basophils cross-linking (see Fig. 5, illustration on the right) is also low. On the other hand, formation of a folate-FITC/anti-FITC IgG complex is prevalent in the circulation of immunized animals (24). Providing that a folate-hapten conjugate maintains its 1:1 ligand-to-hapten stoichiometry, only the presence of multi-haptenated species such as bis-FITC would facilitate the formation of large immune complexes. Admittedly, such type III hypersensitivity would have a delayed effect on the host, whereas allergic reaction to bis-FITC (as a contaminant in folate-FITC) happens immediately following exposure, thereby supporting the hypothesis of an IgE-mediated allergic response. Finally, folate-FITC did not cause any allergic

reaction in non-immunized animals, an observation that excludes the possibility of an anaphylactoid-type reaction (data not shown).

As allergic IgE production is an indication of suboptimal immunogen doses, increasing KLH-FITC dose had an opposite effect on folate-FITC hypersensitivity, and it significantly reduced this risk in mice and guinea pigs. Despite the effort to remove bis-FITC from the folate-FITC product to levels below detection, the residual trace amounts of the contaminating species appeared to be sufficient to trigger the

Fig. 8. Antitumor activity of folate-FITC in the presence of IL-2/IFN- α with or without desensitization. KLH-FITC/GPI-0100-immunized mice were s.c. implanted with 250,000 viable FR-positive 4T1c2 tumor cells. In the first regimen (Fig. 3a), treatment of folate-FITC plus IL-2/IFN- α started 13 days after the third immunization. In the second regimen (Fig. 3b), early folate-FITC desensitization started on the day of the second immunization (day 8), and IL-2/IFN- α were added 3 days after the third immunization. Folate-FITC (500 nmol/kg) and IL-2 (20,000 U) were dosed at five times/week. IFN- α (25,000 U) was dosed at three times/week. Points Mean of five animals; bars SEM. $P > 0.05$ when the group pre-treated with folate-FITC was compared to the group treated with folate-FITC on a regular schedule

allergic reaction in those animals immunized with low doses of KLH-FITC. Because of this, we devised an early desensitization strategy that involved daily dosing of the folate-hapten starting 1 week after the first immunization (i.e., before antibody titers had reached any significant level; Fig. 2b). This strategy was strikingly effective in protecting "under-immunized" animals against folate-FITC hypersensitivity when they were challenged with 10% bis-FITC-spiked folate-FITC product (i.e., an exaggeratingly high impurity level; Fig. 7, Table II). This information suggested to us that the allergic reaction toward a folate-hapten conjugate might be prevented if the agent were repetitively administered prior to antibody generation in order to facilitate a gradual desensitization. Such a technique is in common practice, especially in dealing with IgE-mediated hypersensitivity to low-molecular-weight drugs such as antibiotics, carboplatin, cisplatin, etc. Interestingly, carboplatin desensitization is one example where short desensitization protocols (e.g., less than 6 h) have an unacceptable failure rate in patients with carboplatin allergy, but longer infusion times (over days) is well tolerated without recurrence of the allergic reaction and with good antitumor responses (25).

Immune-based therapies of FR-positive cancers can be pursued with a variety of strategies including administration of anti-FR monoclonal antibodies (MOv18, MOv19, and LK26) (26–28), radioimmunotherapy (29,30), treatment with bispecific ligand/antibodies (31,32), administration of genetically modified T cells (33), and vaccination with DNA (34) and mRNA-transfected dendritic cells (35,36). Here, we offer a unique approach whereby metastatic FR-positive tumors can be non-invasively marked with a folate-hapten conjugate. In a hapten-immunized host, endogenous anti-hapten antibodies are recruited to opsonize the tumor cell leading to Fc receptor-mediated immune attack. A long-term survival benefit may result from dying tumor cells being phagocytosed by local antigen-presenting cells, such as dendritic cells and macrophages, and fragments thereof being presented to T cells for recognition. As published previously, a folate-hapten conjugate may be designed to minimize its potential for allergy by subtle manipulation of the hapten linker chemistry. We have also shown here that increasing the KLH-hapten dose and/or desensitizing early with a folate-hapten product can prevent any potential allergy in a hapten-immunized host. This strategy has also proven to be effective in clinical settings, and folate-FITC was safely administered to KLH-FITC immunized cancer patients in a recent clinical trial (37). It remains important, however, to remove any multivalent hapten species in the folate-hapten preparations, as these species are highly allergenic in actively immunized hosts.

CONCLUSION

Folate-hapten-targeted immunotherapy is currently being evaluated for both cancer and autoimmune/inflammatory diseases such as arthritis (38), lupus (39), and others (reviewed in 40). So far, the efficacy and safety profiles of the folate-targeted hapten therapy in test animals have been encouraging. As with many cancer vaccines that are currently undergoing investigation in the clinic, the therapeutic potential of our strategy is largely dependent on tumor type (immunogenicity) and tumor burden at the time of treatment

initiation. We previously reported that the folate-FITC immunotherapy was more effective against the syngeneic M109 lung carcinoma than the 4T1c2 mammary carcinoma; also, the same treatment was insufficient to induce a significant response against large M109 tumors (>300 mm³) where the tumor doubling time was ~4 days (9). However, we believe that our strategy may prove to be useful in an adjuvant setting in combination with radiation as published previously (9) or with standard chemotherapy and other targeted cancer therapies directed against FR or other unrelated tumor antigens. More importantly, it presents the opportunity to exploit a cancer patient's own immune response against a foreign hapten to destroy otherwise immune-evading cancer tissue as well as effectively "cleanup" microscopic disease and thereby prevent any recurrence of the cancer.

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