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Folate-Targeted Hapten Immunotherapy of Adjuvant-Induced Arthritis: Comparison of Hapten Potencies

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Abstract: We have previously reported that disease symptoms can be greatly ameliorated in rodents with adjuvant-induced arthritis (AIA) by first immunizing the rodents against fluorescein and then treating the animals with folate−fluorescein. In this targeted hapten therapy, folate−fluorescein was shown to decorate folate receptor (FR)-expressing activated macrophages with fluorescein (an immunogenic hapten), leading to binding of antifluorescein antibodies and the consequent elimination of the activated macrophages by Fc receptor-expressing immune cells. In the current study, we compare the therapeutic potencies of a variety of FR-targeted haptens in treating the symptoms of AIA in rats. Rats were immunized with either dinitrophenyl (DNP) or trinitrophenyl (TNP) conjugated to keyhole limpet hemocyanin followed by induction of AIA with heat-inactivated *Mycobacterium butyricum*. Following development of arthritis, rats were treated with one of five folate−hapten conjugates (folate−DNP1, folate−DNP2, folate−DNP3, folate−FITC, or folate−TNP) at two different doses (30 nmol/kg or 200 nmol/kg) 5×/week for 25 days. Symptoms of AIA in treated rats, including paw swelling, arthritis score, splenomegaly, bone erosion, and FR^+ activated macrophage density in inflamed tissues, were quantitated over the course of therapy. Although all folate−hapten conjugates promoted a reduction in disease symptoms, folate−TNP and folate−FITC proved to be more potent than any of the 3 folate−DNP conjugates. We conclude that both folate−TNP and folate−FITC constitute promising haptens for use in FR-targeted immunotherapy of arthritis.

Keywords: Activated macrophages; arthritis; dinitrophenyl; drug-targeting; folate receptor; fluorescein; immunotherapy; trinitrophenyl

Introduction

Rheumatoid arthritis is an autoimmune disease that is accompanied by synovial inflammation, resulting in destruction of cartilage and bone. Although the exact causes of RA remain unknown, activated macrophages are thought to play a pivotal role in its pathogenesis, because they extravasate into affected joints[1,2] and secrete pro-inflammatory cytokines (primarily TNF-α, IL-1, IL-6), metalloproteinases, reactive oxygen species, prostaglandins and chemokines that directly damage the tissue and induce an influx of other inflammatory immune cells.[1−3] Consistent with this scenario, the magnitude of macrophage accumulation and activation is known to correlate with the severity of RA symptoms.[1,2]

Many current rheumatoid arthritis therapies have been developed to suppress pro-inflammatory products of activated

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macrophages. Adalimumab has been designed to neutralize TNF-α.[4] Anakinra inhibits IL-1.[5−7] Celecoxib blocks the activity of cyclooxygenase-2 (COX-2),[8] and tocilizumab has been developed to neutralize the IL-6 receptor.[9] Because each of these therapies reduces the level of a mediator of inflammation, without eliminating its source, symptoms commonly recur when treatment is discontinued.[4,10] In contrast, TNF-α antagonists, etanercept and infliximab, are thought to promote apoptosis in macrophages, thereby expanding their potential therapeutic impact by eliminating the cell responsible for multiple pro-inflammatory stimuli.[11]

Elimination of activated macrophages from synovial tissue has been reported to alleviate arthritis symptoms in two animal models of the disease.[12] Since activated, but not resting macrophages or most other healthy cells, express a folate receptor-beta (FR-β),[13−16] FR-β has been exploited as a target to deplete activated macrophages from diseased joints. For example, a recombinant immunotoxin composed of a truncated form of the *Pseudomonas* exotoxin A conjugated to a monoclonal antibody specific for human FR-β has been shown to kill macrophages from RA synovial fluid.[15,17] However, the immunotoxin also shows severe liver toxicity in animal models of the disease and induces the production of antiexotoxin antibodies, both of which may limit application of the therapy in the clinic.[18]

Our group has explored the use of FR-targeted haptens to promote immune-mediated elimination of FR-expressing cells, including activated macrophages[12] and cancer cells.[19,20] In the case of adjuvant-induced arthritis (AIA), rodents were immunized against fluorescein, a small hapten, using fluorescein isothiocyanate conjugated to keyhole limpet hemocyanin (KLH−FITC). Arthritic animals were then treated with a folate−FITC conjugate to decorate the surfaces of their inflammatory FR^+ macrophages with the fluorescent hapten, resulting in binding of antifluorescein antibodies to the "painted" macrophages and their consequent destruction by Fc receptor-expressing immune cells.[12] Although this targeted hapten therapy significantly reduced disease symptoms in affected animals, only one hapten was ever tested, and its potency relative to other haptens was never explored.

In this study, we compare the immunotherapeutic potencies of several folate−hapten conjugates in treating AIA, including three conjugates of dinitrophenyl (DNP), one of trinitrophenyl (TNP), and the previous conjugate of fluorescein, folate−FITC. We report that the folate conjugate of TNP has a similar therapeutic efficacy in treatment of adjuvant-induced arthritis to folate−FITC, whereas all three conjugates of DNP are of inferior potency.

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Experimental Section

Synthesis, Purification, and Characterization of Folate−DNP and Folate−TNP Conjugates. Picrylsulfonic acid was obtained from Wako Chemicals (Virginia, USA) and 2,4-dinitrophenyl sulfonic acid was purchased from Avocado Research Chemicals Ltd. (Massachusetts, USA). Ethyldiisopropylcarbodiimide, 2,4-dinitrophenylacetic acid and *N*-hydroxysuccinimide were purchased from Aldrich (Missouri, USA). All other chemicals were purchased from major suppliers. Compounds were purified by reverse phase preparative high performance liquid chromatography (HPLC) (Waters, xTerra C_{18} 10 μm; 19 × 250 mm) and analyzed by reverse phase analytical HPLC (Waters, x-bridge C_{18} 5 μm; 3.0 × 15 mm). All the compounds were characterized using a Bruker 500 MHz cryoprobe NMR instrument and Waters LC−MS (ESI) mass spectrometer.

Synthesis of Folate−Hapten Conjugates. Folate-Lys, the major building block of folate−hapten conjugates, was synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide chemistry starting from Fmoc-Lys (*tert*-butyloxycarbonyl)-Wang resin. N^{10} TFA-pteroic acid (N^{10} TFA-Ptc) was synthesized according to a previously described method[21] with slight modifications. For preparation of folate−TNP, folate-Lys was dissolved in 0.1 M NaOH solution and picrylsulfonic acid (TNP) was added. After adjusting the pH to ∼10.5, the reaction mixture was stirred overnight to yield crude folate−TNP. The crude compound was purified by reverse phase preparative HPLC [A (aqueous phase) = 10 mM NH_4OAc (pH = 7.0), B (organic phase) = acetonitrile; λ (wavelength) = 320 nm; solvent gradient: 1% B to 70% B in 25 min, 80% B wash 40 min run] and analyzed by reverse phase analytical HPLC (λ = 280 nm, 330 nm; 1% B to 70% B in 10 min, 80% B wash 15 min run). After removal of acetonitrile under reduced pressure, pure fractions were freeze-dried to yield folate−TNP as a yellow solid (Figure 1). $t_R \sim 7.1$ min (analytical HPLC). ^{1}H NMR (DMSO-d_6/D_2O): δ 1.18 (m, 2H, Pep-H); 1.45 (m, 1H, Pep-H); 1.54 (m, 3H, Pep-H); 1.81 (m, 1H, Pep-H); 1.95 (m, 1H, Pep-H); 2.11 (m, 2H, Pep-H); 2.88 (m, 2H, Pep-H); 3.94 (m, 1H, Lys-αH); 4.12 (m, 1H, Glu-αH); 4.46 (s, 2H, Ptc-H); 6.61 (d, J = 8.5 Hz, 2H, Ptc-Ar-H); 7.56 (d, J = 8.5 Hz, 2H, Ptc-Ar-H); 8.60 (s,1H, Ptc−Ar-H); 8.85 (s, 2H, Ar-H). LC−MS $(M + H)^+$: calcd for $C_{31}H_{33}N_{12}O_{13}$ = 781.7; found = 781.4.

Folate−DNP1, folate−DNP2, and folate−DNP3 were synthesized according to Lu et al., 2007,[20] with only minor modifications (Figure 1), and folate−FITC was a kind gift from Endocyte, Inc. (Indiana, USA).

Relative Binding Affinity Assay of Folate−Hapten Conjugates to Human FR-β (hFR-β). The relative binding affinities of folate−hapten conjugates to hFR-β were examined using a previously described method.[22] CHO-β cells expressing hFR-β were seeded on 48-well plates at 70% confluency and cultured at 37 °C in folate-deficient RPMI1640 medium (Invitrogen, California, USA) supplemented with 1× penicillin/streptomycin (Gibco, California, USA) and 10% fetal bovine serum (FBS) (Atlanta Biologicals, Georgia, USA) in a 5% CO_2 humidified incubator. Twenty-four hours later, cells were washed twice with PBS (pH 7.4), after which a 10 nM solution of ^{3}H-folic acid (GE Healthcare, New Jersey, USA) was added with increasing concentrations (10^{-10} M to 10^{-5} M) of either folate−FITC, folate−DNP1, folate−DNP2, folate−DNP3, or folate−TNP in cell culture medium (Figure 2). Cells were incubated at 37 °C for 1 h and washed 3× with 0.5 mL of PBS. 0.5 mL of 1.0% sodium dodecyl sulfate (SDS) in PBS was added to each well, and after 5 min, cell lysates were collected and transferred to vials containing scintillation cocktail and counted for radioactivity. Relative binding affinity was defined as the molar ratio required for displacement of 50% of bound ^{3}H-folic acid from the cell surface. Relative binding affinity of underivatized folic acid for its receptor was set as 1. Values above or below 1 represent binding affinities of compounds that are higher or lower than that of folic acid, respectively.

Animals and Husbandry. Female Lewis rats (175−200 g) were purchased from Harlan (Indiana, USA). All animal care and use was performed according to NIH guidelines and in compliance with protocols approved by the Purdue Animal Use and Care Committee (PACUC). Rats were kept at 22 °C in a 12 h dark−light cycle. Four weeks prior to immunization, rats were transferred to a folate-deficient rodent diet to normalize the levels of serum folate to the physiological range[12] (Figure 3a).

Immunization and Antibody Titers. Induction of anti-hapten antibodies was achieved according to a previously described method[19] with slight modifications. Rats were immunized sc 3× with 100 μg of either KLH−FITC, KLH−DNP (Biosearch Technologies, California, USA), or KLH−TNP (Biosearch Technologies, California, USA) in PBS containing GPI-0100 adjuvant (Endocyte, Inc., Indiana, USA).[20] Ten days after the last immunization, blood was collected by tail vein puncture, and the serum was analyzed for antibody titers against FITC, DNP, and TNP by an enzyme-linked immunosorbent assay (ELISA).[19] Titers are presented as the dilution where 50% of each antigen is bound.

Induction and Monitoring of AIA in Rats. Experimental adjuvant-induced arthritis was induced according to a previously described method.[12,23] Briefly, adjuvant was prepared by adding finely ground heat-killed *Mycobacterium butyricum* (Difco Laboratories, Michigan, USA) in mineral oil (Sigma-Aldrich, Missouri, USA) at a final concentration of 1 mg/mL. The adjuvant was kept under constant stirring to ensure homogeneous distribution of the mycobacterial particles. Immunized rats were anesthetized with ketamine and

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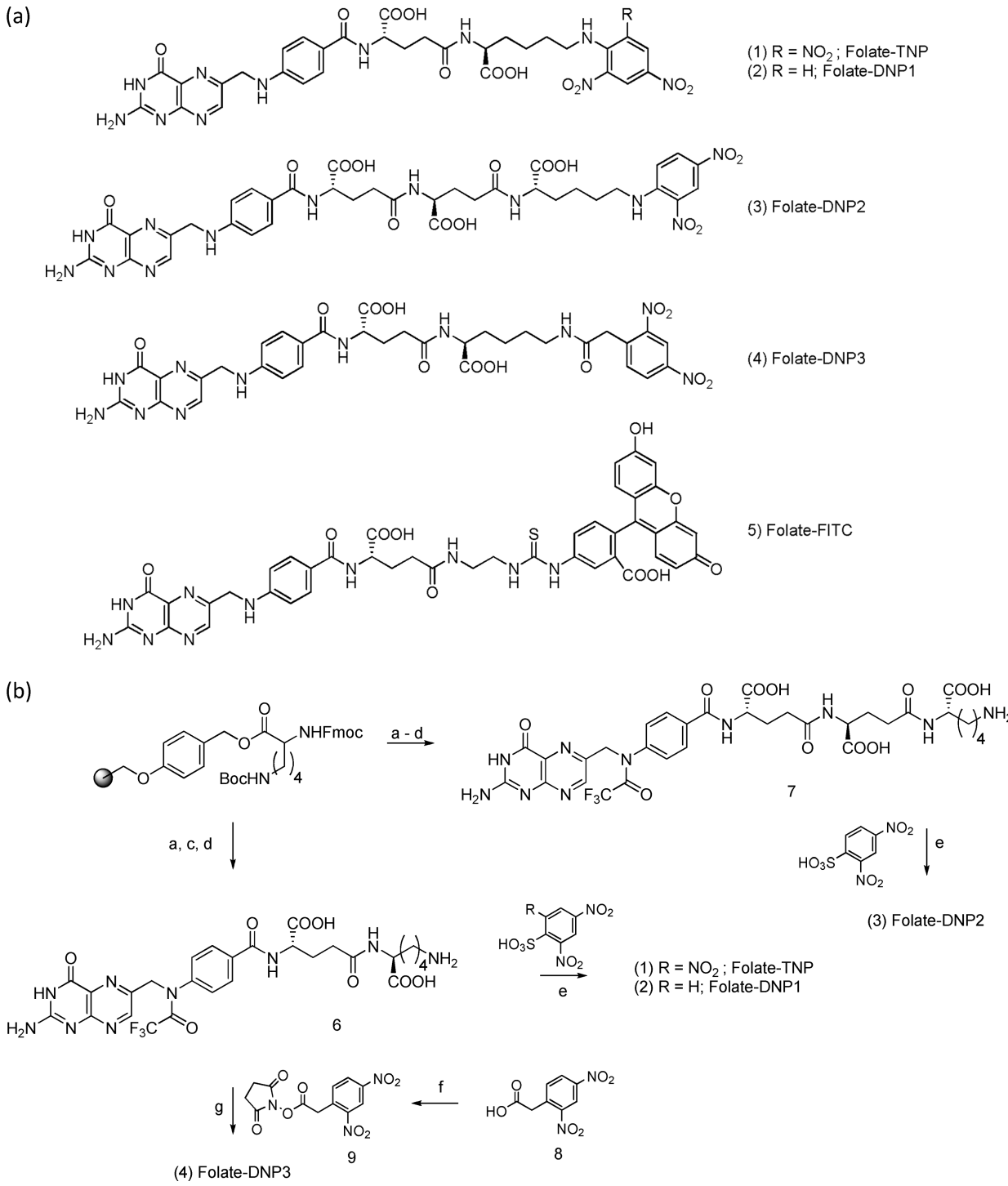

Figure 1. (a) Structures of folate−hapten conjugates and (b) schemes for their synthesis. Reagents and conditions: (a) (i) 20% piperidine/DMF, RT (room temperature), 10 min; (ii) Fmoc-Glu(O^{t}Bu)-OH, HBTU, HOBt, DIPEA, 2 h; (b) (i) 20% piperidine/DMF, RT, 10 min; (ii) Fmoc-Glu(O^{t}Bu)-OH, HBTU, HOBt, DIPEA, 2 h; (c) (i) 20% piperidine/DMF, RT, 10 min; (ii) N^{10}-TFA-Ptc-OH, HBTU, HOBt, DIPEA, 2 h; (d) TFA/H_2O/TIPS (95:2.5:2.5), 1 h; (e) aqueous NaOH (pH 10.5), 24−48 h; (f) DCC, EDC/THF; (g) DIPEA/DMF, RT, 12 h.

xylazine (100 mg/kg and 13 mg/kg, respectively) and injected in the right hind paw with 100 μL of the mycobacterial suspension. Paw inflammation was monitored daily until the first symptoms of RA appeared on the left, noninjected hind paw. At this point, rats were randomly assigned to different treatment groups and treated as described below (Figure 3a).

FR-Targeted Immunotherapy of AIA in Rats. To compare the efficacies of the various folate−hapten conjugates in treating AIA, arthritic rats were injected ip 5×/week with either (1) vehicle alone (PBS), (2) 100 nmol/kg of folate−FITC, (3) 30 nmol/kg folate−DNP1, (4) 200 nmol/kg folate−DNP1, (5) 30 nmol/kg folate−DNP2, (6) 200

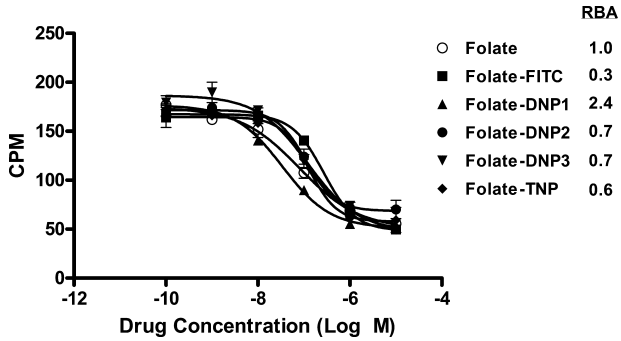

Figure 2. Relative binding affinities of folate−hapten conjugates to hFR-β. CHO-β cells were incubated with 10 nM ^{3}H-folate along with increasing concentrations (10^{-10} M to 10^{-5} M) of (○) folic acid, (■) folate−FITC, (▲) folate−DNP1, (●) folate−DNP2, (▼) folate−DNP3, and (◆) folate−TNP at 37 °C for 1 h. After washing to remove unbound radioactivity, cells were removed from the culture dishes with SDS and radioactivity was measured with a scintillation counter. Data shown are means ± SEM (n = 3). RBA, relative binding affinity. CPM, counts per minute.

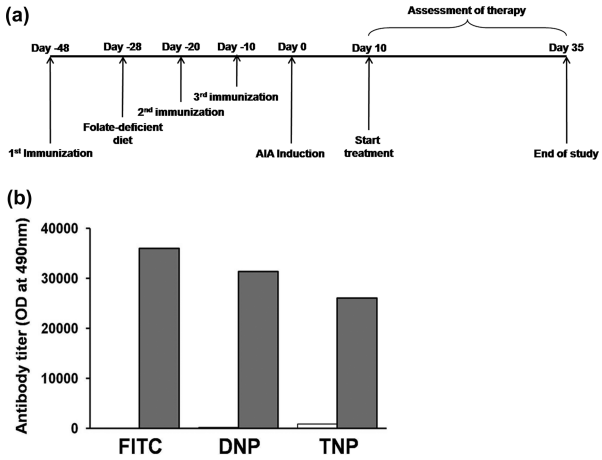

Figure 3. (a) Timetable for immunization and treatment of animals. (b) Determination of antibody titers against FITC, DNP and TNP. Rats were immunized three times with either KLH−FITC, KLH−DNP, or KLH−TNP in a GPI-0100 adjuvant solution. The serum was collected, and antibody titers against FITC, DNP or TNP were determined by ELISA, as described in Immunization and Antibody Titers. Gray bars and open bars represent immune and preimmune antibody titers, respectively.

nmol/kg folate−DNP2, (7) 30 nmol/kg folate−DNP3, (8) 200 nmol/kg folate−DNP3, (9) 30 nmol/kg folate−TNP, or (10) 200 nmol/kg folate−TNP. Paw volumes, arthritis scores, spleen enlargement, bone erosion, and the biodistribution of FR^+ macrophages were then quantitated as a function of time during therapy, as described below.

Paw volumes were measured 2×/week by multiplying length, height, and width of the noninjected hind paw.[12] Arthritis scores were graded on a scale of 0−4 (Chondrex, Inc.) per paw (excluding the injected paw) 2×/week by a trained person blinded to the treatments. Splenomegaly was assessed 25 days after initial treatment by euthanizing the animal and measuring the weight of the resected organs. Bone degradation of the noninjected hind paw was evaluated by X-ray in one representative rat from each hapten group treated at 200 nmol/kg folate−hapten conjugate. The biodistribution of FR^+ macrophages in each treatment group was also quantitated using the FR-targeted radioimaging agent, ^{99m}Tc-EC20, which was prepared as described previously.[24] Briefly, each rat was injected ip with 500 μCi of radioactivity at a dose of 67 nmol/kg of EC20. Four hours later, spleens and livers were dissected, and the radioactivity of the indicated tissues was measured using a γ-scintillation counter. Relative biodistributions of ^{99m}Tc-EC20 were presented as a % injected dose per g of tissue.

Statistical Analysis. Statistical significance among experimental groups was calculated using Student's t-test. Values of $p < 0.05$ were considered significant.

Results

Relative Binding Affinities of Folate−Hapten Conjugates to hFR-β. The relative binding affinities of the various folate−DNP and folate−TNP conjugates were compared by examining their association with FR-β on CHO-β cells. As shown in Figure 2, the binding affinity of folate−DNP1 was 2.4 times higher than that of folic acid, whereas those of the other folate−hapten conjugates were slightly lower (1.3 and 3.3 times). Curiously, the binding affinities of all folate−DNP and folate−TNP conjugates were stronger than that of folate−FITC. The rank order of folate−hapten conjugate affinities was folate−DNP1 > folate−DNP2 > folate−DNP3 > folate−TNP > folate−FITC (Figure 2).

Comparison of Antibody Titers against FITC, DNP and TNP Haptens. For immunotherapy of RA, a high titer of antibodies against the targeted hapten is essential. The titers of rats immunized with KLH−FITC, KLH−DNP or KLH−TNP are illustrated graphically in Figure 3b. While titers were essentially similar, a weak ranking in the sequence of FITC > DNP > TNP was observed (Figure 3b).

Comparison of Potencies of Folate−Hapten Conjugates in Treatment of AIA in Rats. *Induction of AIA in Rats.* To compare the efficacy of the various folate−hapten conjugates in treating arthritis, AIA was induced in rats by injecting a heat-killed mycobacterial suspension into the right hind paw (hereafter termed the injected paw) and disease symptoms were monitored in the noninjected paws. Although severe

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localized swelling of the injected paw was seen within one day, swelling and erythema of the noninjected paws due to systemic inflammation were first observed at ~day 10.

Paw Volumes. One of the diagnostic characteristics of AIA is paw swelling. To compare the potency of the various folate−hapten conjugates in suppressing paw swelling caused by systemic inflammation, volume changes in the noninjected hind paws of arthritic rats were measured during treatment. Because previous dosing studies with folate−FITC (manuscript in preparation) revealed that optimal responses were observed at a daily dose of 100 nmol/kg, all dosing with the new haptens was performed at both 30 nmol/kg and 200 nmol/kg to ensure that a near optimal dose was examined.

Paw swelling was found to be reduced in all hapten-treated groups, while no reduction in paw volume was seen in the PBS-treated control (Figure 4a−d). However, only folate−FITC and folate−TNP demonstrated a statistically significant impact on paw swelling when compared to the PBS-treated controls ($p < 0.05$), and there was no statistical difference in efficacy between folate−FITC and folate−TNP (Figure 4d). Furthermore, the efficacy of a 30 nmol/kg dose was similar to a 200 nmol/kg dose for folate−DNP3 and folate−TNP, but inferior to 200 nmol/kg for folate−DNP1 and folate−DNP2.

Arthritis Scores. The relative potencies of the various haptens in preventing the increase in arthritis score characteristic of the control (PBS-treated) group was TNP = FITC > DNP3 > DNP2 > DNP1 (Figure 4e−h). As noted for paw swelling, arthritic scores were also statistically different from PBS-treated controls only for both concentrations of folate−TNP and the single concentration of folate−FITC tested. There did not seem to be a major impact of folate−hapten dose on arthritis score.

Splenomegaly. Splenomegaly, a consequence of systemic inflammation, constitutes another diagnostic characteristic of arthritis in both humans and rats.[25] To determine whether folate−hapten conjugates suppress splenomegaly, spleen masses were measured and compared among treatment groups. As seen in Figure 5, immunotherapy using each of the targeted haptens led to a similar, statistically significant, suppression of splenomegaly. Thus, spleen masses in all hapten-treated groups increased ~30% compared to those of healthy rats, while spleen masses in the PBS-treated group increased ~80%.

Bone Erosion. RA is also frequently characterized by progressive bone degradation. To examine whether folate−hapten conjugates suppress this bone erosion, bones of noninjected hind paws were analyzed by X-ray at the end of the study. Severe bone erosion was observed in the PBS-treated group, however, bone degradation was not detectable in the groups treated with folate−hapten conjugates (Figure 6).

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Analysis of ^{99m}Tc-EC20 Biodistribution. As previously reported, macrophages become activated and express FR-β in the spleen, liver, and other tissues of arthritic animals.[12] To examine whether treatment with folate−hapten conjugates depletes FR-β^+ activated macrophages systemically, uptake of ^{99m}Tc-EC20, a FR-targeted radioimaging agent that is internalized by FR-β^+ activated macrophages, was quantitated in the above organs. High levels of ^{99m}Tc-EC20 in the spleens and livers were observed in the PBS-treated group; however, uptake of ^{99m}Tc-EC20 was significantly reduced in all groups treated with folate−hapten conjugates (Figure 7). In this analysis, folate−DNP1 and folate−DNP2 appeared to be superior to folate−DNP3 and folate−TNP.

Discussion

We have demonstrated here that multiple FR-targeted haptens can mediate alleviation of the major symptoms of AIA in rats, including paw swelling, arthritis score, splenomegaly, bone degradation, and systemic accumulation of FR$^+$ activated macrophages. Although the efficacies of the different haptens in mediating amelioration of the above symptoms were often similar, folate−TNP and folate−FITC consistently outperformed the three folate−DNP conjugates in suppression of the localized symptoms of arthritis (i.e., paw swelling and arthritis score). Importantly, a comparison of antihapten titers cannot explain this trend, since titers decreased in the sequence FITC > DNP > TNP. Differences in binding affinities also cannot offer an explanation, since the binding affinities decreased in the order folate−DNP1 > folate−DNP2 > folate−DNP3 > folate−TNP > folate−FITC, implying that the conjugates with the lowest affinities contained the targeted haptens with the best therapeutic response. Unfortunately, at present we cannot offer a molecular explanation for the differences in the therapeutic potencies exhibited by the folate−hapten conjugates.

Folate−DNP conjugates have been previously compared for their potencies in eliminating FR-α expressing tumors in murine models of cancer.[20] In this previous study, therapeutic potencies were found to decrease in the sequence folate−DNP1 > folate−DNP2 > folate−DNP3 (folate−TNP and folate−FITC were not examined). Importantly, the affinities of folate−hapten conjugates for the folate receptor isoform expressed on cancer cells (FR-α) also decreased in the above order. Why the therapeutic efficacies of our folate−hapten conjugates did not correlate with their binding affinities for FR-β remains unclear. However, it should be noted that the affinities of folates for FR-β and FR-α can be different.[26,27] Further, the recycling time of FR-α on cancer cells is long (>10 h),[28] whereas the recycling time of FR-β on activated macrophages is very short (~20 min, manuscript in preparation). Consequently, a similar correlation between binding affinity and therapeutic efficacy may not be expected.

In our previous study, it has been observed that treatment of arthritic rats with folate−FITC can reduce the number of

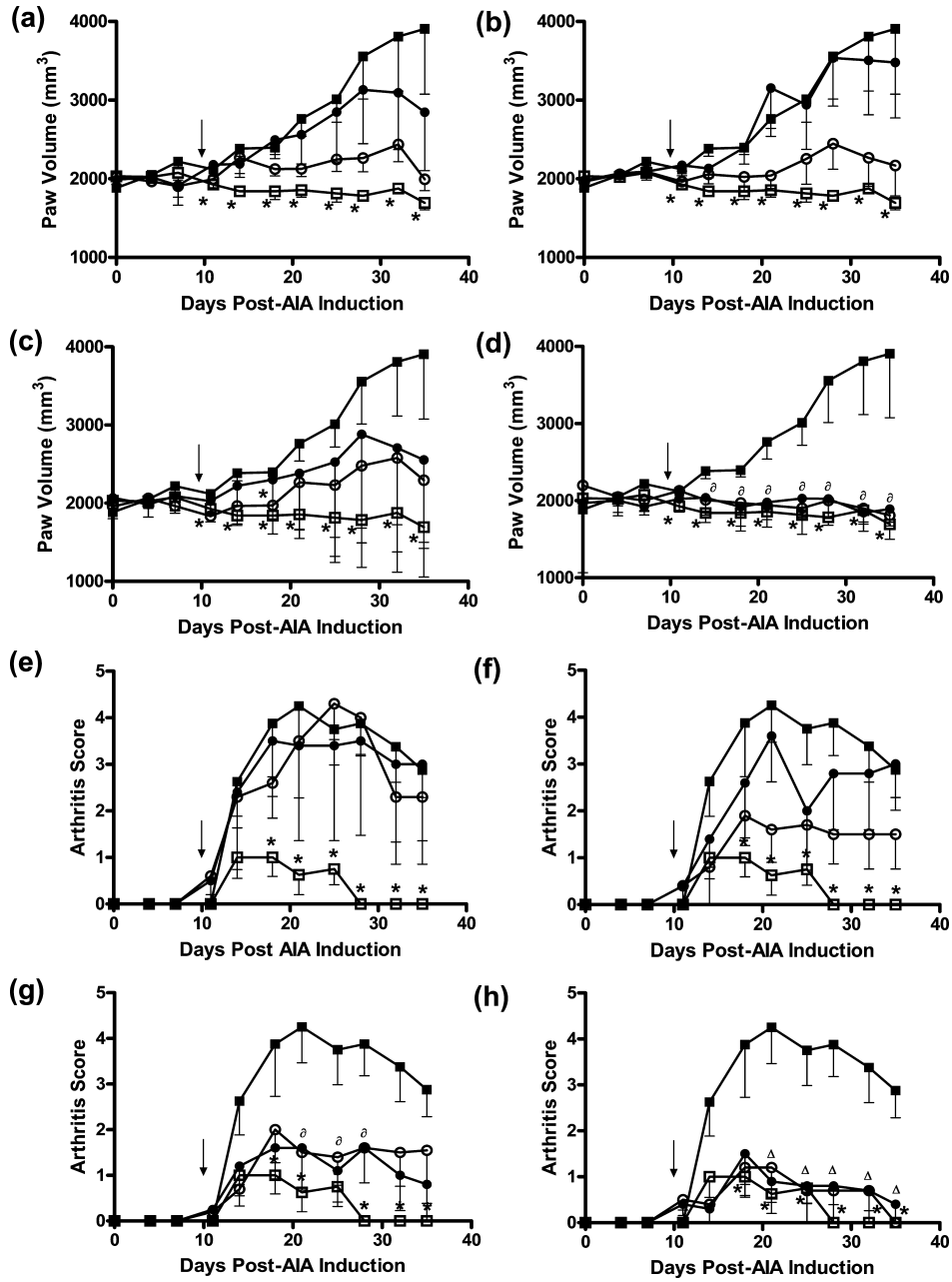

Figure 4. FR-targeted immunotherapy suppresses paw swelling and arthritis scores in rats. Arthritic rats were treated with two different doses: 30 nmol/kg (●) or 200 nmol/kg (○) of each folate−hapten conjugate 5×/week for 25 days. Volume changes in noninjected hind paws of arthritic rats treated with (a) folate−DNP1, (b) folate−DNP2, (c) folate−DNP3, or (d) folate−TNP were measured 2×/week. Arthritis scores of all noninjected paws of rats treated with (e) folate−DNP1, (f) folate−DNP2, (g) folate−DNP3 or (h) folate−TNP were also determined 2×/week. The results of each treatment group are plotted along with the results of PBS- (■) and folate−FITC-treated (□) rats. Data shown are means ± SEM ($n = 5$). Arrow shows time where treatment was started. $^{*}p < 0.05$ compared to PBS; $^{\partial}p < 0.05$ compared to PBS for paw volumes at both concentrations tested of folate−TNP; $^{\Delta}p < 0.05$ compared to PBS for arthritic scores at both concentrations tested of folate−TNP.

FR-β^{+} activated macrophages in the spleens and livers of the treated animals.[12] We have demonstrated here that folate−DNP and folate−TNP conjugates can similarly mediate reduction of FR^{+} activated macrophages from the organs of affected animals. Diminution of systemic inflammation

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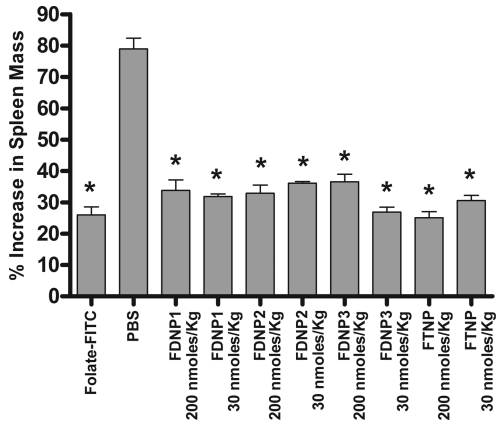

Figure 5. FR-targeted immunotherapy suppresses splenomegaly in arthritic rats. Arthritic rats were treated with each folate−hapten conjugate for 25 days. Spleens were resected and masses recorded. Data are presented as % change in spleen weight relative to the spleen weights of healthy rats. Data shown are means ± SEM ($n = 5$). $^*p < 0.05$ compared to PBS.

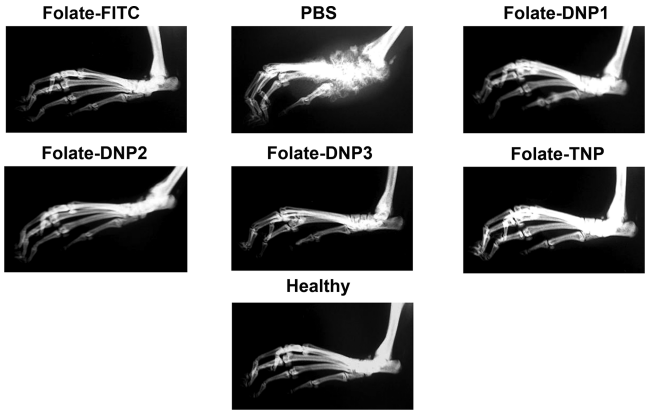

Figure 6. FR-targeted immunotherapy suppresses bone degradation in arthritic rats. At the end of each study, representative rats from control or experimental groups treated with 200 nmol/kg of the various folate−haptens were selected to assess bone degradation in the noninjected hind limb by X-ray radiography.

is important, since systemic inflammation, not localized joint inflammation, is responsible for the increased mortality associated with RA.[29] Thus, while joint pain and stiffness constitute the primary symptoms that trigger a change in patient care, systemic damage to the heart and kidneys etc. is primarily responsible for the reduced life expectancy of RA patients.

Although current arthritis drugs have often proven successful in alleviating disease symptoms, as noted in the

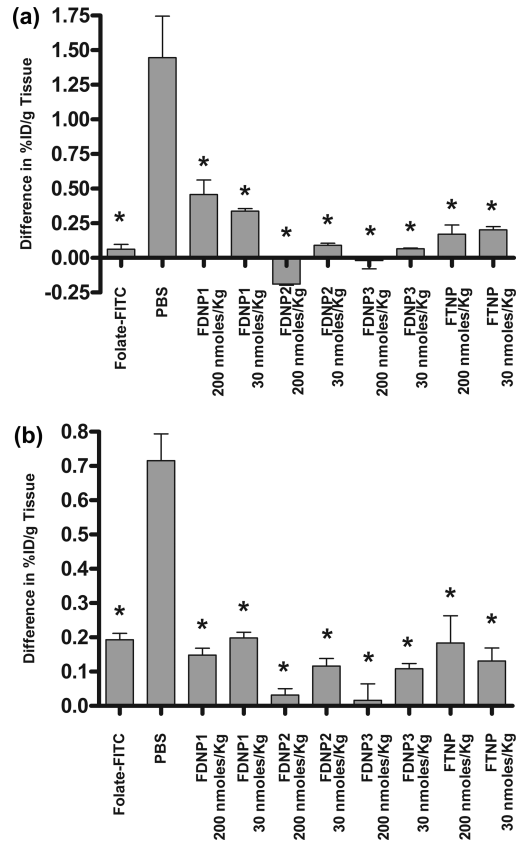

Figure 7. FR-targeted immunotherapy depletes FR^+ activated macrophages in AIA arthritic rats. The content of FR^+ activated macrophages in the (a) spleens and (b) livers of arthritic rats from each group was evaluated by measuring the accumulation of FR-targeted ^{99m}Tc-EC20 by these organs. On the last day of each study, rats from each group were injected ip with ^{99m}Tc-EC20, and after allowing 4 h for unbound ^{99m}Tc-EC20 to clear, animals were euthanized and the indicated organs resected and counted for radioactivity. The *Y*-axis represents the difference in % injected dose of ^{99m}Tc-EC20 per gram of tissue (% ID/g of tissue in liver and spleen of treated rats − % ID/g of tissue in liver and spleen of healthy rats), where ID refers to injected dose. Data shown are means ± SEM ($n = 5$). $^*p < 0.05$ compared to PBS.

Introduction, their therapeutic mechanisms generally target only one of many mediators of the disease.[30−33] FR-targeted immunotherapy, on the other hand, targets and eliminates the cell responsible for most of the inflammatory cytokines, chemokines, metalloproteinases, reactive oxygen species, and prostaglandins (i.e., the activated macrophage) that cause disease symptoms. As a consequence, folate−hapten immunotherapy might be expected to reduce a broader spectrum of disease symptoms than any individual therapy aimed at a single mediator of inflammation. Indeed, a previous comparison of folate−hapten immunotherapy with etanercept, celecoxib, anakinra, clodronate liposomes, and methotrexate confirmed the overall superiority of the FR-targeted immunotherapy over individual commercial therapies.[12]

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Although folate−hapten immunotherapy has not been tested in the clinic for treatment of any inflammatory disease, folate−FITC has been used to treat cancer patients that have folate receptor expressing malignancies (unpublished data). Because a separate set of clinical trials will obviously be required before folate−hapten immunotherapy can be prescribed for inflammatory diseases, it was important to examine whether FITC or another folate−hapten conjugate might be preferred for this application. Our data suggest that either folate−TNP or folate−FITC would constitute an optimal conjugate for folate-targeted immunotherapy of rheumatoid arthritis. And as with selection of cancer patients for folate-targeted immunotherapy of cancer, enrollment of RA patients in folate-targeted immunotherapy for rheumatoid arthritis would be based on demonstration of uptake of a folate-targeted imaging agent, like ^{99m}Tc-EC20, in their inflamed joints.

Although undesired side effects have not been observed in previous studies of our folate−hapten immunotherapy for cancer, RA, and lupus erythematosus,[19,20,28,34−37] the elimination of activated macrophages could increase an individual's susceptibility to infectious disease. Thus, the activated macrophage is known to play a role in resistance to pathogenic microbes, and a potential for immune suppression arises in their absence. While sufficient studies of animals in pathogen-containing environments have not yet been conducted, if an opportunistic infection were to arise, the immunotherapy could be terminated. Moreover, since the infiltration of new monocytes into sites of inflammation is a dynamic process that occurs very quickly,[38] and because FR-negative (resting) macrophages are not eliminated by FR-targeted therapies (personal observations), new activated macrophages should be rapidly generated upon exposure to proinflammatory cytokines. Consequently, a full complement of activated macrophages should quickly emerge upon removal of the folate−hapten conjugate.

In summary, we have demonstrated that various folate conjugates of nitrophenyl haptens, especially folate−TNP, constitute good elicitors of arthritis immunotherapy by elimination of systemic FR^+ activated macrophages. The therapy appears to be well tolerated with no signs of systemic or localized toxicity, and disease symptoms resolve within several days of initiation of treatment.

Abbreviations Used

DNP, dinitrophenyl; FITC, fluorescein isothiocyanate; FR, folate receptor; AIA, adjuvant-induced arthritis; TNP, trinitrophenyl.

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