Folate-Targeted Imaging of Activated Macrophages in Rats With Adjuvant-Induced Arthritis

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Objective. To determine whether overexpression of the high-affinity folate receptor (FR) on activated macrophages can be exploited to selectively target imaging agents to sites of inflammation in rats with adjuvant-induced arthritis (AIA).

Methods. Folic acid was conjugated to a 99mTc chelator (the complex termed EC20), and its distribution was visualized using gamma scintigraphy in healthy rats, rats with AIA, and arthritic rats that had been depleted of macrophages. To confirm that uptake was mediated by the FR, excess folic acid competition studies were conducted, and tissue FR levels were quantitated using a radioligand binding assay. Flow cytometry was also used to investigate uptake of folate conjugates into macrophages of both arthritic and healthy rats.

Results. EC20 concentrated in the arthritic extremities of diseased rats but not in the extremities of healthy rats. The intensity of images of affected tissues was greatly reduced in the presence of excess competing folic acid. The livers and spleens of arthritic animals also showed enhanced uptake of EC20 and increased levels of FR. Depletion of macrophages from arthritic animals reduced tissue FR content and concomitantly abolished uptake of EC20. In addition, macrophages isolated from livers of rats with AIA exhibited a significantly higher binding capacity for folate conjugates than did macrophages obtained from healthy rats.

Conclusion. Although EC20 is currently undergoing clinical evaluation for use in the imaging of ovarian carcinomas, the present results suggest that it may also be useful for assaying the participation of activated macrophages in inflammatory processes such as rheumatoid arthritis.

The folate receptor (FR) is a 38-kd glycosyl phosphatidylinositol–anchored protein that binds the vitamin folic acid with high affinity (Kd < 1 nM) (1). Following receptor binding, rapid endocytosis delivers the vitamin to the inside of the cell, where it is unloaded in an endosomal compartment at low pH (2). Importantly, covalent conjugation of small molecules, proteins, and even liposomes to folic acid does not alter the ability of the vitamin to bind the FR; therefore, folate–drug conjugates can readily enter cells by receptor-mediated endocytosis (3–5).

Because most cells use an unrelated reduced folate carrier to acquire the necessary folic acid (6), expression of the FR is restricted to a few cell types (1). With the exception of the kidney and placenta, normal tissues express low or undetectable levels of FR (1). However, many malignant tissues, including ovarian, breast, bronchial, and brain cancers, express significantly elevated levels of the receptor (7). In fact, it is estimated that 95% of all ovarian carcinomas overexpress the FR (8), highlighting folate conjugation as a possible strategy for the selective delivery of attached imaging and therapeutic agents to ovarian tumors (9).

It was previously reported that FRβ, the nonepithelial isoform of the FR, is expressed on activated (but not resting) synovial macrophages (10). Activated synovial macrophages are present in large numbers in arthritic joints, where they play an active role in rheumatoid arthritis (11). Therefore, we hypothesized that folate derivatization might be exploited to target drugs to cells involved in destructive inflammation. Because the number of activated macrophages in human arthritic joints correlates with the level of articular destruction

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and with poor disease prognosis (11,12), we also envisioned that use of a folate-linked imaging agent might permit visual assessment of the progressive nature of the disease, perhaps enabling a better analysis of optimal treatment regimens.

Scintigraphic imaging agents are a million times more sensitive than magnetic resonance imaging contrast agents, and their selectivity can be enhanced by targeting them to lesion-specific cell markers (13). Indeed, the radioisotope 99mTc has been delivered to arthritic tissues using nonspecific IgG (14,15), anti-CD4 antibodies (16), CD11b/CD14–glycolipopeptide ligands (17,18), and E-selectin–binding peptides (18). Preclinical studies with such radioimaging agents have clearly emphasized the value of imaging arthritic tissues in vivo; however, the selectivity of the current imaging agents is not yet optimal (13), and none of the present compounds is targeted exclusively to activated macrophages. In view of the emergence of FR activity during macrophage activation, we attempted to evaluate whether folate-targeted 99mTc might be used to image activated macrophages associated with arthritis in vivo.

MATERIALS AND METHODS

Materials. EC20 (a folate-linked chelator of 99mTc), EC28 (the same 99mTc chelate complex without folate), and folate–fluorescein isothiocyanate (FITC) were all generous gifts from Endocyte (West Lafayette, IN). Heat-killed Mycoplasma butyricum was purchased from BD Biosciences ( Sparks, MD). Folic acid, light mineral oil, clodronate, collagenase A, and streptavidin–R-phycoerythrin were obtained from Sigma (St. Louis, MO), and Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Gibco BRL (Gaithersburg, MD). Tritium (3H)-labeled folic acid was obtained from American Radiolabeled Chemicals (St. Louis, MO), and Microcon-30 membranes were purchased from Millipore (Bedford, MA). RK4–biotin and ED2–R-phycoerythrin antibodies were acquired from Bachem Biosciences (Philadelphia, PA) and Accurate Chemical & Scientific (Westbury, NY), respectively.

Animal model of arthritis. Arthritis was induced in 150–200-gm female Lewis rats (Charles River, Wilmington, MA), as previously described (19). Briefly, on day 1, rats were anesthetized with ketamine and xylazine hydrochloride, and 0.5 mg of heat-killed M butyricum suspended in mineral oil (5 mg/ml) was injected into the left hind foot. Disease was allowed to progress for 21 days, and the animals were weighed on a daily basis to ensure the status of their health. Arthritis developed in all treated animals, as evidenced by dramatic swelling in the injected paw, progressive swelling in all uninjected limbs, and radiographic analysis of affected limbs. Standard commercial rat diets were not used, because they contain excessive amounts of folic acid that maintain rodent serum folate levels at supraphysiologic concentrations. Rather, all rats were maintained on a folate-deficient diet (Dyets, Bethlehem, PA) for 3 weeks prior to each study, in order to lower serum folate levels to normal physiologic concentrations. Control rats were also maintained on a folate-deficient diet, but arthritis was not induced in these animals. The number of rats used in each study is specified in the legend of the relevant figure.

Elimination of endogenous macrophages. Evaluation of macrophage-independent uptake of the folate-linked imaging agent was accomplished by killing endogenous macrophages with liposomal clodronate. Liposomes, prepared according to a modification of the procedure described by Love et al (20), were formed by rehydrating a thin film of egg phosphatidylcholine (60 mole %) and cholesterol (40 mole %) in an isotonic clodronate solution (250 mg/ml). Small unilamellar vesicles were then generated by extrusion of the liposomes 10 times through a 100-nm polycarbonate membrane using a 10-ml thermobarrel extruder (Lipex Biomembranes, Vancouver, Canada). Unencapsulated clodronate was removed by overnight dialysis through a SpectraPor 300,000-Mr cutoff cellulose acetate membrane ( Spectrum Laboratories, Rancho Domingues, CA), and the clodronate concentration in the retained liposomes was determined as described elsewhere (21). Seventeen days after induction of arthritis and 3 days before administration of the imaging agent (EC20), rats destined for macrophage depletion received a single intraperitoneal injection of liposomes containing 20 mg of clodronate. Macrophage depletion was performed in 6 rats: 3 for biodistribution analysis, and 3 for determinations of FR level.

Scintigraphy and biodistribution analysis. Twelve hours before administration of the imaging agent, all animals received 5 ml of normal saline subcutaneously to ensure proper excretion of unbound imaging agent. Twenty-one days after induction of arthritis, rats were injected intraperitoneally with 500 μCi (2.3 nmoles/rat) of either EC20, EC20 plus a 500-fold molar excess of folic acid, or EC28. Four hours later, rats underwent either gamma scintigraphic imaging or biodistribution analysis. For scintigraphy, 2 separate experiments were conducted, each with 5–10 rats per group. Two experiments were also conducted for biodistribution analysis, each with 3–4 rats per group. Each experiment also contained 1 group of healthy (nonarthritic) rats as a control.

For scintigraphic evaluation, rats were anesthetized with ketamine and xylazine hydrochloride and positioned in ventral recumbency on the image-acquisition surface. Image acquisition was performed for 1 minute at a rate of 50,000–75,000 counts per minute using a Technicare Omega 500 Sigma-410 radioisotope gamma camera (Diagnostix Plus, Garden City Park, NY). Following whole-body imaging, gamma emission from the abdomen and thorax was shielded using 1/8-inch lead plates, and images of the hind limbs were obtained. All data were analyzed using a Medasys MS-DOS-based computer equipped with Medasys Pinnacle software (Medasys Digital Systems, Geneva, Switzerland).

For biodistribution analysis, rats were killed by intraperitoneal injection of sodium pentobarbital. Liver, spleen, heart, lungs, intestine, and kidneys were then harvested, and the level of radiation in each tissue was determined using a gamma counter (Packard BioScience, Meriden, CT).

Assay of tissue FR levels. Tissue FR levels were determined by measuring isolated membrane protein binding to radiolabeled folic acid. Briefly, tissues were homogenized, and cell membranes were isolated by centrifugation. Membrane proteins were solubilized overnight, transferred into a Microcon-30 filtration device, and incubated with 50 nM of
3H-labeled folic acid. A duplicate of each sample, used to determine nonspecific binding, was also exposed to 50 nM of 3H-labeled folic acid but in the presence of 1,000-fold excess, unlabeled folic acid. After unbound 3H-labeled folic acid was washed through the membrane, membrane protein with bound 3H-labeled folic acid was recovered and counted in a scintillation counter (Packard BioScience) to determine the number of active FRs per gram of tissue.

Identification of the FR-expressing cell type in liver. Arthritic and healthy rats were first anesthetized with ketamine

Figure 1. Scintigraphic images of tissues from and extremities of a healthy rat and 2 rats with adjuvant-induced arthritis (AIA). On day 21 of AIA, arthritic and healthy rats were injected with 0.5 mCi of EC20 (a folate-targeted imaging agent). In some cases, rats were co-injected with 500-fold excess folic acid, which acted as a competitive inhibitor for binding to the folate receptor. Four hours later, anesthetized rats were placed in ventral recumbency, and gamma camera images from the ventral surface were acquired. Bodies were oriented with heads pointing toward the top and tails extending toward the bottom of the image. Images of hind limbs were acquired similarly, following lead shielding of the middle and upper body. These images are representative of those obtained from 2 separate experiments, each involving 3 groups (healthy rats, arthritic rats, and arthritic rats treated with excess folic acid). The first experiment included 10 rats per group, and the second experiment included 5 rats per group.
and xylazine hydrochloride, and then a midline incision was
made, starting in the lower abdomen and extending through
the thoracic cavity. A 24-gauge catheter was inserted into the
hepatic vein, and a 24-gauge needle was inserted in the left
ventricle to serve as an outlet for the perfusion fluid. Normal
saline was perfused through the catheter, followed by collage-
nase A solution (0.05% in Gey’s balanced salt solution). Each
solution was delivered for 2 minutes at a rate of 20 ml/minute.
Immediately after perfusion, livers were removed, and the
membrane capsules were dissected away. The remaining gelat-
inous tissue was suspended in collagenase A solution (0.025%
in DMEM) and incubated at 37 °C for 2 hours in the presence
of 1 μM of FITC-folate or 1 μM of FITC-folate plus 1 mM of
folic acid. Cells were then washed 3 times to remove unbound
FITC-folate and immediately prepared for flow cytometry.

Flow cytometry sample preparation and analysis. Liver cell preparations, which had been exposed to FITC-
folate, were treated with ammonium chloride lysis buffer (150
mM of NH₄Cl, 10 mM of KHCO₃, 1 mM of EDTA [pH 7.4])
for 10 minutes at 4°C to lyse red blood cells. Following 3
washes with phosphate buffered saline, the remaining cells
were incubated for 1 hour at 4°C with either ED2–R-
phycoerythrin–labeled mouse anti-rat macrophage antibody,
or RK4–biotin–labeled mouse anti-rat granulocyte antibody
(22). Cells were then washed 2 more times, and those that had
received the biotinylated primary antibody were incubated
with streptavidin–R-phycoerythrin for an additional 30 min-
utes. Following 2 final washes, cells were examined for FITC
and phycoerythrin dual color staining on a FACScan Coulter
XL flow cytometer.

RESULTS

Folate-targeted imaging of arthritic rats. To de-
termine whether folate might be exploited to target
⁹⁹ᵐTc to sites of arthritic inflammation, EC20, a folate-
linked chelator of ⁹⁹ᵐTc, was administered intraperito-
neally to rats, and scintigraphic images were acquired with
a gamma camera. Because of the rapid clearance of
unbound EC20, excellent contrast was obtained at 4
hours postadministration (Figure 1). Importantly,
whole-body uptake, as evaluated by a certified veteri-
nary radiologist, appeared much more intense in ar-
thritic rats than in healthy rats, and the intensity of this
signal was greatly reduced when EC20 was administered
together with a saturating dose of free folic acid (Figure
1). As will be described below, these same results were
obtained when a group of similarly treated rats were
dissected and counted for organ-specific radioactivity.
These data, therefore, suggest that uptake of EC20 by all tissues is determined primarily by a folate-specific receptor.

Intense organ uptake of EC20 prevented visualization of limbs in whole-body images of the arthritic rats. However, images of hind limbs could be easily acquired when middle-body and upper-body emission was shielded. Using such shielding, arthritic limbs were shown to display much greater EC20 uptake than that displayed by healthy extremities, and this uptake was completely eliminated in the presence of excess free folic acid (Figure 1). Furthermore, the left hind foot of the arthritic animals, where inflammation was most severe, displayed greater uptake compared with the less severely affected right hind foot (Figure 1).

Based on the whole-body images, it could be concluded that abdominal organs were responsible for a majority of EC20 uptake in the arthritic animals. To confirm this assessment, liver, spleen, and kidney were removed and imaged separately (Figure 2). Livers of arthritic rats demonstrated the highest uptake of EC20, and livers of healthy rats displayed minimal uptake. Only spleens taken from arthritic rats could be visualized. As expected, kidneys also demonstrated elevated uptake of EC20. This may be attributed to the brush border cells of the proximal convoluted tubules, which express high levels of FR$_1$/H$_9251$ (the high-affinity epithelial isoform of the receptor) for reabsorption of folic acid (23). Free folic acid completely blocked EC20 uptake in liver and spleen; however, the free vitamin only partially decreased uptake by the kidney.

Effects of macrophage depletion. In order to determine whether macrophages might be responsible for the uptake of EC20, resident macrophages were eliminated from arthritic rats using a liposomal clodronate preparation similar to those previously shown to eliminate macrophages from liver, spleen, and synovial membrane of rats with adjuvant-induced arthritis (AIA) (24,25). After clodronate treatment, evaluation of paw size revealed that the paws of clodronate-treated rats were 35–45% smaller than those of untreated rats, which is consistent with previously reported results (25). To determine whether macrophage elimination would influence uptake of the folate-linked imaging agent, EC20 biodistribution analysis was then performed on the clodronate-treated rats and compared with the same analysis of both healthy rats and arthritic rats not treated with clodronate. As shown in Figure 3, depletion of macrophages decreased liver uptake of EC20 $\sim$20-fold in arthritic rats, while retention in the spleen and intestine was reduced by a factor of 3. In most tissues, clodronate treatment depressed EC20 uptake even below levels observed in healthy rats (Figure 3), confirming the hypothesis that activated macrophages account for most of the EC20 retention in normal tissues. In contrast, kidney uptake of EC20 was elevated in rats depleted of macrophages, most likely because the decreased internalization of EC20 by activated macrophages rendered more EC20 available for binding to kidney FRs.

FR-mediated uptake of EC20 in arthritic tissues. Two additional biodistribution studies were conducted to confirm that EC20 uptake by tissues of arthritic rats is mediated by the FR. First, the biodistribution of EC20 was examined both in the presence of and in the absence of a 500-fold excess of free folic acid. As shown in Figure 4, almost complete elimination of EC20 uptake was observed in all tissues except kidney, indicating that binding was indeed mediated by the FR. In fact, excess folic acid competitively reduced retention of EC20 in liver, spleen, heart, lung, intestine, and blood to near background levels (Figure 4). Second, to confirm the role of folate in EC20-mediated targeting of the chelated $^{99m}$Tc, the biodistribution of EC28 (the same complex lacking a folate moiety) was also examined. As displayed in Figure 4, uptake of EC28 was negligible in all tissues except kidney, where retention of the untar-
geted complex was similar to that of EC20 in the presence of competing folic acid.

**FR expression in various tissues of arthritic rats.**

The results described above suggest that the FR is responsible for tissue uptake of EC20. In order to confirm this, we attempted to directly quantitate the level of folate-binding protein in various rat tissues. As anticipated, active FR could be detected in each of the major organs examined, and FR levels were significantly increased in arthritic rats (Figure 4). In addition, FR content correlated well with uptake of EC20 as demonstrated in the biodistribution studies. In fact, the FR assay revealed roughly equivalent levels of receptor in liver and spleen of arthritic animals, in accordance with the similar uptake of EC20 by the same organs (Figure 4). Significantly, systemic elimination of macrophages by clodronate treatment lowered FR levels in liver and spleen from arthritic animals (Figure 5), which is also in good agreement with the EC20 biodistribution analysis. Finally, the FR assay confirmed that neither induction of arthritis nor clodronate treatment alters the levels of FR in kidney or heart, where FR is not thought to be associated with activated macrophages.

**Expression of a functional FR on hepatic macrophages of arthritic rats.** To further confirm that increased uptake of EC20 in livers of arthritic rats is due to

Figure 4. Levels of folate receptor-mediated targeting in tissues from healthy rats and rats with adjuvant-induced arthritis (AIA). On day 21 of AIA, rats (3 per group) were injected intraperitoneally with 0.25 mCi of EC20 (black bars), EC20 plus 500-fold excess folic acid (light gray bars), or EC28 (dark gray bars), and were killed 4 hours later. Tissues were harvested, and percent injected dose of EC20 per gram of tissue was determined by gamma counting. Values are the mean ± SD and are representative of 2 experiments involving 3–4 rats in each of 3 groups.

Figure 5. Levels of folate receptor in tissues from healthy rats and rats with adjuvant-induced arthritis. Internal organs were removed from arthritic rats (black bars), healthy rats (light gray bars), or arthritic, macrophage-depleted rats (dark gray bars) immediately following euthanasia. Samples were prepared, and assays were performed to determine the level of functional folate receptor, as described in Materials and Methods. Samples were obtained from 3 different rats in each of the 3 groups. Values are the mean ± SD.
that only resident tissue macrophages (and clearly only a subpopulation of those) express FR in the liver.

**DISCUSSION**

Activated macrophages are thought to be intimately involved in the pathogenesis of rheumatoid arthritis (11). Activated macrophages directly destroy articular tissue by secreting metalloproteinases and by attracting or activating other immune cells via the release of cytokines (26). The quantitation of activated macrophages in joint tissues might consequently be of diagnostic value, because activated macrophage content correlates well with articular destruction and poor disease prognosis in humans (11,12). Because FR expression may coincide with macrophage activation (10), it seemed reasonable to question whether arthritic joints might be imaged using folate-derivatized imaging agents. The present studies in rats with AIA demonstrate that folate can indeed selectively target $^{99m}$Tc to activated macrophages in vivo, and that folate-linked imaging agents can facilitate the noninvasive analysis of inflammatory activity in situ.

Gamma scintigraphy in rats receiving EC20 demonstrated that arthritic appendages can be readily imaged using folate-targeted $^{99m}$Tc. In contrast, the extremities of healthy rats could not be visualized, demonstrating the selectivity of the imaging agent for arthritis-related applications. Although the intensities of internal organs also increased in AIA, interference from such tissues did not appear to compromise the methodology, because gamma radiation from internal organs could be easily shielded. The fact that excellent contrast can be achieved within 1–2 hours of EC20 injection further suggests that administration of the imaging agent, gamma scintigraphy, and analysis of the image can, conceivably, be completed during the same examination. Taken together, these results suggest that folate-linked imaging agents warrant further scrutiny as possible tools for evaluating arthritis.

It was not unexpected that the liver and spleen (organs that harbor large numbers of macrophages) might also be targeted by EC20 in arthritic animals. In fact, systemic activation of macrophages in rats with AIA has been documented (27). Nevertheless, it was still considered important to establish the specific participation of macrophages in the increased uptake of EC20, because a folate-targeted imaging agent had not previously been examined in arthritic animals. Three experiments were conducted for this purpose.

First, clodronate-loaded liposomes were used to systemically deplete macrophages from the treated rats. Not only were the resulting levels of FR in tissue greatly reduced, but uptake of EC20 in the macrophage-rich organs was nearly eliminated, suggesting that resident macrophages can indeed account for both FR expression and EC20 retention in the reticuloendothelial system organs.

Second, liver cells were disaggregated by collagenase A treatment, and individual cells were evaluated for uptake of folate conjugate. As shown in Figure 6, the vast majority of cells testing positive for uptake of folate conjugate also sorted positive for the rat macrophage marker, ED2, confirming that FR is indeed present on the macrophages.

Finally, because the number of other immune and myelocytic cells is known to be elevated in tissues of rats with AIA (28,29), it was conceivable that still another extravasating blood cell type might be involved in the uptake of EC20. However, neither liver-infiltrating granulocytes nor any blood cell in circulation displayed any capacity to bind FITC-folate. This latter
result was not unexpected, because the β isoform of FR, which was previously shown to be expressed on both hematopoietic progenitor cells and the more mature cells of the monocytic/myelocytic lineages (30,31), was also demonstrated to be incapable of binding folic acid in these cells (31). Consequently, activated macrophages would seem to be the predominant cell type internalizing folate conjugates in the organs of arthritic rats. Perhaps the efficacy of methotrexate (an antifolate) in the treatment of rheumatoid arthritis relates to the overexpression of FR by activated macrophages (10).

It was somewhat surprising to observe that up to 30% of the liver macrophages in healthy rats also expressed the FR (Figure 6). Because a functional FR is not found on resting synovial macrophages (10), it is tempting to speculate that the FITC-folate–binding fraction in the healthy rats might also constitute an activated population. Two observations may support this conjecture. First, activated macrophages are also found in healthy tissues following exposure to immune stimulants and foreign antigens. Given the role of the liver in clearing foreign substances from the body, low-level activation of resident macrophages does not seem unreasonable. Second, the FITC-folate (and EC20)–binding population of liver cells increased significantly upon induction of localized inflammation and systemic macrophage activation (13). Once additional markers for activated rat macrophages become available, it will be important to confirm that uptake of folate conjugates in the healthy livers is also mediated by activated macrophages.

With the ability to exploit folate to deliver attached molecules to activated macrophages now established, the question of any possible future utility naturally arises. Although considerable research on the characteristics of macrophage FRs must still be conducted, it is nevertheless conceivable that folate-linked imaging agents might allow the early development or continued progression of rheumatoid arthritis to be assessed. Because graft-versus-host disease (32), multiple sclerosis (33), Crohn’s disease (34), ulcerative colitis (35), psoriasis (36,37), osteomyelitis (38), and even atherosclerosis (39) may also be caused or aggravated by activated macrophages, it is even possible that the diagnosis and evaluation of these diseases could be aided by a folate-linked imaging/contrast agent. Finally, because therapeutic as well as imaging drugs can be similarly targeted by folate derivitization, a plethora of remedies for the abovementioned pathologic processes might be also considered.

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REFERENCES


