Mutations in the Reduced Folate Carrier Gene Which Confer Dominant Resistance to 5,10-Dideazatetrahydrofolate*

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5,10-Dideazatetrahydrofolate (DDATHF)1 is the prototypical folate antimetabolite inhibitory to de novo purine synthesis (2). Previous studies have demonstrated that the step of de novo purine synthesis affected by (6R)-DDATHF is the first folate-dependent enzyme of this pathway, glycaminide ribonucleotide formyltransferase (GARFT)2 (3, 4). In the accompanying article (1), we describe the characteristics of an unusual cell line, the L1210/D3, that was originally selected for resistance to (6R,6S)-DDATHF. This cell was highly resistant to (6R)-DDATHF, but only in culture conditions under which cellular folate metabolism is supported by folic acid in the medium; during growth on folic acid, L1210/D3 cells are actually more sensitive to (6R)-DDATHF than were the wild-type mouse L1210 cells from which they were derived. We demonstrated that there is an alteration in the membrane transport of folic acid in the L1210/D3 cells, which causes a substantial expansion of the cellular pool of folate cofactors, and that this expanded folate pool blocks the metabolism of (6R)-DDATHF to its polyglutamates.

In the present article, we prove that the reduced folate carrier (RFC) transport system is responsible for the drug-resistant phenotype of the L1210/D3 cell and that the substrate specificity of this transporter in the resistant cells is subtly modified by two point mutations in the RFC gene. Transfection experiments reveal that each mutation contributes part of the phenotype, and that the resistance can be transferred to either cells lacking a functional reduced folate transport system or to wild-type L1210 cells. That is, these transfection experiments demonstrate that the point mutations in the RFC gene are causative of the phenotype of the L1210/D3 cells and that this phenotype represents a genetically dominant trait.

EXPERIMENTAL PROCEDURES

Materials—Monensin and methotrexate were purchased from Calbiochem (San Diego, CA) and Sigma, respectively. (6R)-DDATHF and chemically synthesized (6R)-DDATHF polyglutamate standards were kindly provided by Dr. Chuan Shih of Eli Lilly Research Laboratories. (6R)-[3H]DDATHF was prepared by coupling [3,4-3H]glutamic acid with 5,10-dideazapteroic acid as described in the accompanying article (1). [3,5,7,9-3H]Methotrexate, (6S)-[3,5,7,9-3H]5-formyltetrahydrofolate, and [3,5,7,9-3H]folic acid were purchased from Moravek Biochemicals (Brea, CA).

Selection of L1210 Sublines—Mycoplasma-free mouse L1210 cells were passaged in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum in the presence of increasing concentrations of (6R,6S)-DDATHF until the culture resumed the same growth rate as a parallel culture maintained without drug. This process was repeated at progressively higher drug concentration until a cell line capable of growth in 3 μM (6R,6S)-DDATHF was obtained; this line, which was denoted as L1210/D3, was then cloned in soft agarose and frozen. The characteristics and derivation of L1210/D3 cells and of L1210/D0.5, a cloned intermediate cell line capable of growth in 0.5 but not 3 μM (6R,6S)-DDATHF, are described in detail in the accompanying article (1). L1210/A cells (5) had been isolated by selection of parental L1210 cells with methotrexate and were cloned by limiting dilution; they were kindly provided for these experiments by Dr. I. David Goldman of Albert Einstein Cancer Center as was a line of mouse erythroleukemia (MEL) cells which overexpress folate-binding protein isotype β (FBP-β) (6).

Growth Inhibition Studies—Exponentially growing cells were transferred to drug-containing medium in 24-well plates at an initial density of 1.0 × 104 cells per well. Significant differences were noted between cell lines after 72 h of drug exposure; cultures were further incubated in drug-free medium for 3 days before harvest. The 100% growth control for each cell line was determined by plating drug-free cultures at various initial densities, and the relative drug sensitivity of each line was expressed as a percentage of this control. For each drug concentration, drug sensitivity was the mean of triplicate determinations and expressed as the concentration of drug required to reduce cell growth to 50% of control.

5,10-Dideazatetrahydrofolate (DDATHF) is the prototypical folate antimetabolite inhibitory to de novo purine synthesis (2). Previous studies have demonstrated that the step of de novo purine synthesis affected by (6R)-DDATHF is the first folate-dependent enzyme of this pathway, glycaminide ribonucleotide formyltransferase (GARFT)3 (3, 4). In the accompanying article (1), we describe the characteristics of an unusual cell line, the L1210/D3, that was originally selected for resistance to (6R,6S)-DDATHF. This cell was highly resistant to (6R)-DDATHF, but only in culture conditions under which cellular folate metabolism is supported by folic acid in the medium; during growth on folic acid, L1210/D3 cells are actually more sensitive to (6R)-DDATHF than were the wild-type mouse L1210 cells from which they were derived. We demonstrated that there is an alteration in the membrane transport of folic acid in the L1210/D3 cells, which causes a substantial expansion of the cellular pool of folate cofactors, and that this expanded folate pool blocks the metabolism of (6R)-DDATHF to its polyglutamates.

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of 2 × 10^5/ml in a total volume of 1.5 ml. Culture density was determined after 72 h at 37 °C (N) and compared with the density of wells containing each of the two mutations found in the L1210/D3 cells (see "Results") were separable, due to the different sizes caused by the presence or absence of a tag separating the mutants.

Northern Analysis—RNA from cell lines was extracted using the Triazol reagent (Life Technologies, Inc., Gaithersburg, MD), denatured BamHI these primers (underlined) are preceded by an aliquot of this cDNA using 3 units of reverse transcribed cDNA was purified on a GlassMAX cartridge essential for construction and directional cloning of cDNAs containing each of the two mutations found in the L1210/D3 cells (see below). The PCR conditions used after an initial denaturation at 93 °C for 3 min, consisted of four cycles of 94 °C denaturation for 1 min, 52 °C annealing for 1 min, and 4 min of extension at 72 °C, followed by 30 cycles in which the annealing temperature was raised to 58 °C, and finally a 5-min extension at 72 °C. A 1800-bp PCR fragment which spanned nt −14 to 1765 of the published mouse rfc cDNA was gel purified and isolated on glass powder, then ligated into the pCRII vector (Stratagene). Individual colonies were picked, colonies with an 1800-bp fragment which contained the remainder of the cDNA for the RFC coding region, including the codon 105 mutation found in L1210/D3 cells. These two fragments were gel-purified and one fragment from L1210/D3 cells was mixed with the opposite fragment from L1210 cells and the mixtures were used in triple ligations into pcDNA III, allowing the construction of identically cloned expression plasmids containing each of the two mutations one at a time. The regions of each mutation and restriction endonuclease digestion were sequenced in each construct to confirm their identity.

The pcDNAIII constructs were not found to allow good expression in the L1210 cell, so that, subsequently, the cloned RFC constructs were released from the pcDNAIII vector with BamHI and XhoI, the fragments were gel isolated, treated with Klenow fragment, and ligated into the tk-PGK vector (13) which had been linearized with EcoRI. This vector expressed the cloned E. coli strain MC1061/P3 were transfected with these plasmids, and the orientation of the insert was verified by restriction endonuclease digestion and a subsequent sequence analysis.

Isolation of Cloned Transfectant Cell Lines—The function of wild-type and mutant RFC proteins was studied in clonal cell lines constructed from L1210 and L1210A cells transfected with the corresponding cDNAs. Exponentially growing recipient cells were harvested and washed once with serum-free medium, then resuspended at a final density of 1 × 10^6 cells/tube in 0.8 ml of DEAE-dextran buffer containing 40 μg of plasmid DNA and 50 μg of pTK-PGK or with the same plasmid containing an insert representing wild-type, double mutant, or single mutant rfc cDNAs. The suspensions were subjected to electroporation at 250 V and 350 microfarads, following which each transfection mixture was brought to 10 ml with RPMI 1640 medium containing 10% serum, and allowed to recover at 37 °C for 36 h. Each culture was then adjusted to 1 × 10^6 cells/ml in complete medium containing 0.75 mg/ml active G418 and 20 μg 2-mercaptoethanol, and 20,000 cells were distributed into each well of five 96-well plates. For each transfection, cell growth occurred in 10–15 of these wells after 8–10 days; cells from multiple wells were expanded for further analysis. Transfectants constructed from L1210A cells were screened first for their ability to grow in 0.2 μM MTX and cultures which proved sensitive to MTX in this test were judged to express the transfected rfc gene. For each series, RNA was prepared from 6–10 such cultures and the levels of expression of the endogenous and transfected rfc genes and glyceraldehyde-3-phosphate dehydrogenase were determined by Northern blotting and quantitation with a Molecular Dynamics PhosphorImager. Both endogenous and exogenous rfc transcripts could be quantitated in these screens and were easily distinguished from each other by the differences in the levels of expression of the endogenous 3′- and 5′-untranslated regions. Transfections into L1210 cells were, likewise, distributed into wells at 20,000 cells/well, and outgrowing wells were expanded and screened for rfc expression

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levels by Northern blot without prescreening for function. For each transfection, 2–6 cultures were selected which did not express more than three times the amount of exogenous rfc transcripts than the amount of wild-type rfc transcript found in L1210 cells. Cell lines were prepared from these cultures by cloning G418 resistant and low level RFC expressing cultures by limiting dilution: an average of 0.1 cell was prepared from these cultures by cloning G418 resistant and low level RFC expressing cultures by limiting dilution: an average of 0.1 cell was

RESULTS

Pharmacological Definition of the Transport System Involved in the Phenotype of the L1210/D3 Cell Line—Translocation of folate derivatives across the plasma membrane of mammalian cells can be mediated at neutral pH by at least three transport systems: 1) a carrier-mediated system commonly known as the RFC (15–17); 2) a low pH optimum transport system (18); and 3) a series of glycosylphosphatidylinositol-linked folate-binding proteins, which facilitate the intracellular transfer of folates via an endocytotic-like mechanism (19). Northern blot analysis revealed that L1210/D3 cells, like the parental L1210 cells, when grown in micromolar concentrations of folic acid, express only trace levels of transcripts for the folate-binding protein-α and -β isotypes (Fig. 1). However, the reduced folate transport protein encoded by the rfc gene was expressed in the L1210/D3 cells, as it was in the wild-type leukemic cells (Fig. 1C). No differences in the sizes of these transcripts were detected, but the abundance of the rfc transcript in L1210/D3 cells was about 50% of that in wild-type L1210 cells when corrected for RNA loading (n = 3).

The distinguishing characteristics of the RFC are a marked substrate preference for methotrexate and reduced folates (15) which efficiently block transport of any alternative substrates by this transport route, whereas the folate receptor-mediated endocytotic pathway has been demonstrated to be highly sensitive to inhibition by the ionophore monensin (19). When these criteria were applied to the transport of folic acid in L1210/D3
cells, it was found that transport of folic acid over a 2-min period was \(\sim 90\%\) inhibited by 20 \(\mu M\) methotrexate (5–7 times the previously described Michaelis constant for MTX transport in L1210 cells (15–18)), but that monensin at 10 \(\mu M\) had little effect (\(\sim 10\%\) inhibition) on folate transport in either parental L1210 cells or L1210/D3 cells.

Influx Kinetics for Folate Compounds in L1210/D3 Cells—
There were only small differences in the influx kinetics of (6R)-DDATHF, MTX, and 5-formyltetrahydrofolate in L1210/D3 cells, compared with the kinetics observed in parental L1210 cells (Table II). The \(K_m\) for transport of (6R)-DDATHF was 3.5 times higher in L1210/D3 cells, but there was a small change in \(V_{max}\), resulting in a decrease in overall of about 2.5-fold in the first order rate constant of transport (\(k_r\)). Because the rate of transport of (6R)-DDATHF in L1210/D3 cells would be made equivalent to that in L1210 cells by an increase in extracellular concentration of 2.5-fold, this alteration could not explain the 400–500-fold resistance (1) of these cells. In L1210/D3 cells, \(V_{max}\) for the transport of MTX was identical to that found in L1210 cells, but the \(K_m\) for transport appeared to be about two-thirds that in L1210 cells. Likewise, kinetics of the uptake of 5-formyltetrahydrofolate measured over 60 s in the parental and resistant cell lines showed small differences, with about a 2-fold higher \(K_m\) in L1210/D3 cells.

The major change observed was a dramatic increase in the transport of folic acid in L1210/D3 cells. The transport of folic acid by L1210 cells was slow, and the saturation kinetics needed to estimate a \(K_m\), or a \(V_{max}\) were not observed at achievable concentrations of extracellular radiolabeled folic acid (Fig. 2). In contrast, folic acid was transported into L1210/D3 cells with clear saturation kinetics, and had a \(V_{max}\) for transport equivalent to or higher than that of the best substrates for the reduced folate carrier system (Table I). Others (9) have estimated the \(K_m\) for folic acid transport in L1210 as 600 \(\mu M\), a value which would be indirectly supported by several reports that folic acid interferes with the transport of methotrexate in L1210 cells with a \(K_i\) of 200–400 \(\mu M\) (9, 15, 20). The inhibition of (6R)-DDATHF transport by folic acid was analyzed by Dixon analysis; the \(K_i\) with which folic acid inhibited (6R)-DDATHF transport was 340 \(\mu M\) in L1210 cells and 18 \(\mu M\) in L1210/D3 cells. The initial slope of a plot of transport velocity versus extracellular folic acid (Fig. 2) allowed an estimate of the first order rate constant (\(k_r\)) for transport in L1210 cells (Table I). Comparing this value with that found for L1210/D3 cells, it appeared that the \(k\) for transport of folic acid had increased by about 100-fold in the mutant cells. In agreement with the concept of a more efficient transport of folic acid in L1210/D3 cells compared with L1210 cells, the concentration of folic acid at which L1210/D3 cells grew half-maximally was found to be \(15 \pm 1\) \(\mu M\), compared with a value of 280 \(\pm 40\) \(\mu M\) for L1210 cells.

Although it was clear that a massive difference in the transport of folic acid had been selected for during isolation of these resistant cells, it could not be determined from such transport studies alone whether a transporter other than the folate receptor family members or the reduced folate carrier had been overexpressed or whether the characteristics of one of these carrier systems had been subtly altered. However, the most likely system involved seemed to be the reduced folate carrier.

To address this point directly, cDNA corresponding to RFC transcripts were cloned and sequenced.

Isolation and Sequencing of the rfc Gene in Mutant L1210 Cells—Poly(A\(^{-}\))-selected RNA from L1210 cells and L1210/D3 cells was reverse transcribed and an 1800-bp fragment which spanned the coding region of the rfc gene (12) was amplified by PCR and cloned. Seven clones were isolated from L1210/D3 mRNA and three from L1210 mRNA; each was completely sequenced. Several randomly positioned point mutations were detected in the cloned PCR products from both L1210 cells and L1210/D3 cells, each of which was present in only one cloned PCR product; these were interpreted as Taq polymerase-induced misincorporations. However, two point mutations were detected in each of the seven cloned PCR isolates from L1210/D3 cells which were absent from the three cloned PCR products isolated from L1210 parental cells. Codon 48 contained a point mutation at nt 142\(^2\) which changed the ATC

### Table I

| Transport Substrate | \(K_m\) \(\mu M\) | \(V_{max}\) pmol/10\(^6\) cells \(\times\) min | \(k_r\) | \(K_m\) \(\mu M\) | \(V_{max}\) pmol/10\(^6\) cells \(\times\) min | \(k_r\) |
|---------------------|-------------------|---------------------------------------------|--------|-------------------|---------------------------------------------|--------|
| DDATHF (3)          | 1.7 ± 0.6         | 0.93 ± 0.14                                 | 0.55   | 5.7 ± 0.16        | 1.23 ± 0.12                                 | 0.22   |
| MTX (2)             | 3.3 ± 0.1         | 0.53 ± 0.02                                 | 0.16   | 2.0 ± 0.1         | 0.60 ± 0.04                                 | 0.30   |
| Folic acid (2)      | (340)\(^6\)       | Not measurable\(^b\)                         | \(\sim 5 \times 10^{-4}\) | 19.7 ± 2.9    | 1.20 ± 0.27                                 | 0.061  |
| 5-Formyltetrahydrofolate (2) | 3.5 ± 1.1 | 0.64 ± 0.14                                 | 0.18   | 6.3 ± 1.4        | 0.45 ± 0.08                                 | 0.071  |

\(^a\) \(k_r\) represents the first order rate constant for transport, defined as \(V_{max}/K_m\), and is in units of (pmol/10\(^6\) cells \(\times\) min) \(\mu M\). \(^b\) Below the \(K_m\) for transport, the rate of entry into the cells would be represented by \(k_r\) \(\times\) substrate concentration, and hence, \(k_r\) reflects the efficiency of transport at low concentrations of each substrate.

\(^b\) The transport of folic acid into L1210 cells did not saturate at achievable concentrations of folic acid in L1210 cells, disallowing measurement of either \(K_m\) or \(V_{max}\) in these experiments. The value listed in parentheses for \(K_m\) was actually a \(K_i\) derived by a Dixon analysis of the inhibition of DDATHF transport by folic acid; for comparison, the \(K_i\) value for folic acid in L1210/D3 cells was 18 \(\mu M\). The \(k_r\) value listed was estimated from the initial slope of \(v\) versus \(S\) plots such as Fig. 2.
encoding an isoleucine to a TTC encoding a phenylalanine. Likewise, nt 313 of the coding region was mutated from a T to G, resulting in the substitution of a glycine for a tryptophan at codon 105. All seven PCR clones from L1210/D3 cells had both mutations, indicating that two separate mutation events had accumulated in this single gene during the multistep selection of the L1210/D3 cell. Because every clone sequenced bore these mutations, it appeared that either the genome of the L1210/D3 cells contained only the mutant allele, i.e. that the wild-type allele had been deleted or converted, or that the wild-type allele was still present but had been silenced.

**Sequencing of the Genomic DNA from L1210 and L1210/D3 Cells**—The region of the *rfc* gene immediately around each of these two mutations was isolated from genomic DNA in PCR reactions that straddled an intron (14), to assure that the products obtained were unique and not representative of cDNA contaminants. In these reactions, a single 3.4-kilobase PCR fragment was obtained, somewhat larger than the 2.9-kilobase size expected from the placement of the forward PCR primer (about 100 nt from the end of exon 2) and that of the reverse primer (about 180 nt into exon 3) and the reported (14) size of the intervening intron 2 in mouse DNA (2.6 kilobase). However, the correct identity of the amplified region was verified by strong hybridization of the PCR fragment with a *rfc* cDNA probe on Southern blots which were washed to very high stringency (0.2 × SSC at 70 °C). The amplified regions of exons 2 and 3 would be expected to (and did) contain *rfc* codons 48 and 105, respectively. When the PCR fragments were directly sequenced, only the wild-type sequence was obtained at codons 48 and 105 in the L1210 cell control (Fig. 3, left sequences). For the genomic PCR fragment from L1210/D3 cells, however, the sequence found at the position of codon 48 was both TCC and ATC (Fig. 3A, right sequence), and nt 313 showed both a T and a G (Fig. 3B, right sequence), proving that both wild-type and double mutant *rfc* alleles were present in genomic DNA from L1210/D3 cells. We concluded that the wild-type *rfc* allele had been selectively silenced in L1210/D3 cells, presumably by epigenetic mechanisms.

**Genotype of the L1210/D0.5 Cell Line**—A cell line capable of continuous growth in the presence of 0.5 μM (6R,6S)-DDATHF which represented an intermediate step in the development of the L1210/D3 cell had been cloned and frozen (1). The genotype of L1210/D0.5 at codons 48 and 105 in the L1210 cell control (Fig. 3, left sequences) for the genomic PCR fragment from L1210/D3 cells, however, the sequence found at the position of codon 48 was both TCC and ATC (Fig. 3A, right sequence), and nt 313 showed both a T and a G (Fig. 3B, right sequence), proving that both wild-type and double mutant *rfc* alleles were present in genomic DNA from L1210/D3 cells. We concluded that the wild-type *rfc* allele had been selectively silenced in L1210/D3 cells, presumably by epigenetic mechanisms.

*rfc Specificity Altered by Point Mutations*

by Northern analysis for expression of the *rfc* gene. Clones which expressed no more than 3-fold higher levels of mRNA than found in L1210 cells were selected for study. The RFC-null L1210/A cells were highly resistant to (6R)-DDATHF (Fig. 4A and Table II), as expected, but were more sensitive to this drug than L1210/D3 cells. Transfection of the cDNA for the *rfc* gene isolated from wild-type L1210 cells into L1210/A cells resulted in cloned transfectant cell lines which were as sensitive to (6R)-DDATHF as L1210/D3 cells (Fig. 4A and Table II). This was the case for several cloned cell lines representing presumably different sites for the integration of cDNA into the mouse genome, and a spectrum of levels of expression of mRNA ranging from equivalent to the endogenous to about 3 times higher levels (data not shown). On the other hand, transfection of the cDNA isolated from L1210/D3 cells, containing the point mutations in codons 48 and 105, into L1210/A cells resulted in the isolation of cloned transfectant cells which had the same sensitivity to (6R)-DDATHF as L1210/D3 cells (Fig. 4A and Table II). Transfection of cDNAs which contained only one mutant codon (either the mutation in codon 48 or the mutation in codon 105) into L1210/A cells revealed that each point mutation conferred partial resistance to (6R)-DDATHF (Table II). Mutation at codon 48 imparted a higher level of resistance to DDATHF than did the mutation at codon 105. Hence, we concluded that the point mutations in codons 48 and 105 of the *rfc* cDNA were causative of the resistance to DDATHF in L1210/D3 cells and that each mutation individually had conferred partial resist-
ance during the multistep process of selection of these resistant cells.

Characterization of Transfectant Cell Lines—The transport of (6R)-DDATHF and folic acid was studied in the cloned transfectant cell lines. The rate of transport of both (6R)-DDATHF and folic acid was somewhat variable in L1210/A cells that had been transfected with the wild-type rfc, presumably reflecting differences in the level of expression of the transfected cDNAs (Table III). However, the ratio of transport of (6R)-DDATHF to that of folic acid was remarkably similar among cloned transfectant cell lines and reflected the ratio found in parental L1210 cells. Transfection of the cDNA containing both codon 48 and codon 105 mutations resulted in a substantial increase in the transport of folic acid, and the preference for transport of folic acid and (6R)-DDATHF approached that found in mutant L1210/D3 cells (Table III). Hence, the alteration in substrate preference caused by the codon 48 mutation and by the codon 105 mutation (Table III) paralleled the level of resistance induced by each single mutation (Table II). Other studies demonstrated that the expression of the double mutant RFC protein resulted in the expansion of the cellular folate pool similarly to that found in the L1210/D3 cell (1) (data not shown).

Transfection of rfc cDNAs into Cells Expressing the Wild-type

| Table II
| Growth inhibition of L1210 cell transfectants by DDATHF |
|---------------------------------|-----------------|-----------------|
| Recipient cell | Transfected cDNA | Cell line | IC_{50} for DDATHF |
|----------------|-----------------|-----------------|-------------------|
| L1210          |                |                | 23 ± 3            |
| L1210/D0.5     |                |                | 3100 ± 300        |
| L1210/D3       |                |                | 9000 ± 1000       |
| L1210/A        |                |                | 1700 ± 520        |
| L1210/A I48F   |                |                | 6200 ± 500        |
| L1210/A I48F   |                |                | 7800 ± 2300       |
| L1210/A W105G  |                |                | 6300 ± 1890       |
| L1210/A I48F   |                |                | 1950 ± 500        |
| L1210/A I48F   |                |                | 2000 ± 1          |
| L1210/A W105G  |                |                | 470 ± 120         |
| L1210/A W105G  |                |                | 450 ± 15          |

| Table III
| Transfer of altered transport characteristics by transfection |
|---------------------------------|-----------------|-----------------|
| Cell line | Transport rate (pmol/10^{6} cells × min) | Transport preference (DDATHF/folic acid) |
|----------------|-----------------|-----------------|
| L1210          |                |                | 36.5            |
| L1210/D0.5     |                |                | 25.7            |
| L1210/D3       |                |                | 31.5            |
| L1210/A        |                |                | 26.4            |
| wt-A 01        |                |                | 51.4            |
| wt-A 07        |                |                | 33.5            |
| wt-A 14        |                |                | 33.2            |
| wt-A 16        |                |                | 1.8             |
| Mean = 35.7    |                |                | 1.8             |
| I48F-A 02      |                |                | 2.8             |
| W105G-A 12     |                |                | 6.6             |

Reduced Folate Carrier—Wild-type and mutant rfc cDNAs were transfected into parental L1210 cells to determine whether the L1210/D3 phenotype could be transferred to cells expressing normal RFC. When the rfc cDNA isolated from L1210 cells was transfected into wild-type L1210 cells, the increased expression of the wild-type rfc gene in the transfectants resulted in either no change or small changes in sensitivity to (6R)-DDATHF (Fig. 4B and Table IV). Transfer of a rfc cDNA containing the I48F and W105G mutations into wild-type L1210 cells transferred substantial levels of resistance to (6R)-DDATHF (Fig. 4B and Table IV). However, the resistance
of transfectants expressing the double mutant rfc gene in a background of wild-type L1210 cells was significantly less than seen in transfection of this same cDNA into RFC null L1210/A cells. Hence, the mutant rfc gene conferred a dominant trait, but the intensity of the phenotype was attenuated by the expression of the wild-type RFC protein.

**DISCUSSION**

In this article, we present evidence that the two point mutations identified in the rfc gene are the cause of the resistance to DDATHF seen in the L1210/D3 cells. The proof of this statement rests on the demonstration that expression of a cDNA encoding the I48F,W105G double mutant rfc into RFC null L1210/A cells recapitulates the phenotype of the L1210/D3 cells. The RFC species altered by these mutations recognizes folic acid as a good substrate, and the enhanced transport of folic acid causes an expansion of the intracellular folate pool and a subsequent restriction of the ability of FPGS to metabolize DDATHF (1). The exact mechanism by which the expanded folate pool controls the polyglutamation of (6'R)-DDATHF is not yet clear, but we presume that it is by a direct effect on the FPGS protein.

Preclinical (21) and clinical therapeutic trials (22, 24) have shown that the toxicity of (6'R)-DDATHF to animals and man can be substantially decreased by the co-administration of oral folic acid. Animal studies have shown that the toxicity of (6'R)-DDATHF can be shifted by a surprising 1000-fold by the administration of rather small doses of oral folic acid to animals on a low folate diet (21). We propose that this effect may be the same or closely related to the effect we herein demonstrate. Thus, small doses of folic acid might expand the pool of intracellular folates in drug-sensitive stem cells, thereby preventing polyglutamation of (6'R)-DDATHF, as demonstrated in the L1210/D3 cell in this and a companion article (1), consequently preventing drug toxicity.

The route whereby folic acid enters the cell in standard culture medium containing 2.3 μM folic acid remains uncertain, and somewhat controversial. It is commonly taken that the reduced folate transporter does not efficiently transport folic acid, and certainly not at pH 7.4 (15, 18). Metabolic inhibitors and competitors for methotrexate transport have little effect on folic acid transport (9, 18), and mammalian cell mutants which have substantial decreases in the $V_{\text{max}}$ for transport of methotrexate still transport folic acid (9). Both of these lines of evidence suggest that folic acid passes the cell membrane using a transport system other than the reduced folate carrier. Likewise, the L1210/A (5) cell appears to lack all rfc function, yet transports folic acid sufficiently to allow normal growth in standard cell culture medium. However, we note that transfection of the wild-type rfc cDNA into L1210/A cells substantially enhanced the rate of transport of folic acid in some lines of cloned transfectants (Table III, lines wt-A01 and wt-A07), an indication that the RFC can, indeed, translocate folic acid.

The major finding in this study was that each of two point mutations in the rfc gene modified the substrate specificity of this system and allowed facile transport of folic acid. Accurate transport kinetics could not be measured for folic acid in L1210 cells due to the slow transport rates and poor saturability of this system at achievable extracellular concentrations of labeled folic acid. However, the introduction of the I48F and W105G mutations markedly increased folic acid influx, with an estimated 20-fold reduction in the $K_m$ and an independently estimated $\sim 100$-fold increase in $k'_l$ for folic acid with only minor changes in the kinetics of transport of (6'R)-DDATHF, MTX, and 5-formyltetrahydrofolate. Hence, point mutations in the rfc gene can impart quite dramatic changes in the substrate specificity of this transport system. Amino acids 48 and 105 must constitute part of the substrate recognition site for the RFC and, as such, are likely to be close together in the folded protein and to be solvent-exposed on the exterior surface of the membrane transporter. Codons 48 and 105 correspond to regions of the RFC protein predicted to lie within the first and third transmembrane domains (12). Other recent reports have implicated these regions as critical to transporter function. A point mutation in codon 130, which is predicted to be in the middle of the third transmembrane domain of the RFC, markedly impaired carrier mobility without a change in the affinity of carrier for MTX (13). In addition, a point mutation in codon 46 resulted in MTX resistance due to a highly selective change in the mobility of the carrier loaded with folate substrates without a change in substrate binding (23).

From our analysis of the characteristics of transfectant cell lines expressing cDNAs constructed to have only one of the rfc mutations found in L1210/D3 cells, it was clear that both the W105G and I48F mutations contributed to the phenotype of the L1210/D3 cell. Interestingly, each altered the specificity of the RFC substrate recognition domain in the same manner, at least at the level of detail that we have analyzed to date. Of the level of drug resistance observed in the double mutant transfectants (Table II), most was caused by the I48F mutations. Thus, whereas the transfectants expressing the I48F,W105G double mutant rfc were 300-fold resistant to DDATHF, those expressing the I48F single mutant rfc were 85-fold resistant to drug, and transfectants expressing the W105G single mutant rfc were 20-fold resistant. We presume that each mutation in one allele of the rfc gene emerged in the mass L1210 culture at a single step of drug selection to result in the L1210/D0.5 cell line. Thereafter, the second, wild-type allele became silenced to yield the more highly resistant L1210/D3 cell in which only the double mutant rfc was being expressed. We are currently analyzing the mechanism which induced the differences between these two cell lines in detail to clarify the validity of this hypothesis.

It was somewhat surprising that the L1210/D3 cell expressed only the mutant allele of the rfc gene. The analysis of genomic DNA from L1210/D3 cells indicated that a wild-type allele for this gene was present; hence, the wild-type allele appears to be silenced in these cells, either by methylation or by mutations.

TABLE IV

**Transfer of the resistant phenotype into wild type L1210 cells by transfection**

| Recipient cell | Transfected cDNA | Cell line | $IC_{50}$ for DDATHF $n$ |
|---------------|----------------|-----------|-------------------------|
| L1210         | wt-rfc         | L1210     | 23 ± 3                  |
| L1210/D0.5    | wt-rfc         | L1210     | 3100 ± 300              |
| L1210/D3      | wt-rfc         | L1210     | 9000 ± 1000             |
| L1210         | I48F,W105G-rfc | L1210     | 49 ± 4                  |
| L1210         | I48F,W105G-rfc | L1210     | 9000 ± 1000             |
| L1210         | I48F,W105G-rfc | L1210     | 3030 ± 580              |
| L1210         | I48F,W105G-rfc | L1210     | 660 ± 190               |

3 J. Roberts, R. Tombes, B. Mitchell, and R. G. Moran, submitted for publication.
affecting the promoter. This is a clear example of the selection of a phenotype under maintained pressure of a drug in which one allele of a gene is first mutated, then the other silenced. This has enormous impact on our conception of the origin of drug resistance, for it suggests that the frequency of drug resistance may often be determined by the spontaneous mutation at a single allele, followed by the as yet unmeasured rate of gene silencing of the normal allele under selection. The L1210/A cell line has previously been shown to result from a mutation in one allele of the rfc gene and an apparent silencing of the other allele (13).

Finally, it should be noted that the I48F,W105G double mutated rfc cDNA represents a new dominant selectable marker which can be used for selection in gene transfer experiments, somatic cell genetics or, indeed, gene therapy approaches. Transfection into the parental L1210 cells shifted the sensitivity of transfectants to (6R)-DDATHF, although the concentration of drug which inhibited these transfectants were noticeably lower than that of the L1210/D3 cells. Thus, the expression of the endogenous wild-type RFC protein modifies the effect of expression of the dominant mutant gene, an effect that would be predicted by the biochemical mechanism of resistance.

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