Identification and Characterization of a Mutation in the Dihydrofolate Reductase Gene from the Methotrexate-resistant Chinese Hamster Ovary Cell Line Pro-3 MtxR™

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A methotrexate-resistant Chinese hamster ovary cell line (Pro-3 MtxR™), resistant due to a low-level amplified, altered target enzyme, dihydrofolate reductase (DHFR), has been characterized on the molecular level. The cDNA and coding regions of all six DHFR exons were amplified in vitro using Taq polymerase and directly sequenced. Analysis of the Pro-3 MtxR™ DHFR cDNA demonstrated a C → T base transition at nucleotide 67 that results in the substitution of phenylalanine for leucine at residue 22 and the loss of a BsaI site in the Pro-3 MtxR™ cDNA. This mutation results in a decreased binding of methotrexate to the altered enzyme. Molecular modeling of Leu²² → Phe supports the concept of the importance of Leu²² in the active site of the enzyme and indicates that replacement with phenylalanine will decrease the binding of methotrexate.

The enzyme dihydrofolate reductase (DHFR, 1 5,6,7,8-tetrahydrofolate: NADP⁺ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, an essential carrier of one-carbon units in the biosynthesis of thymidylate, purine nucleotides, and methyl compounds. DHFR is the target enzyme for a number of pharmacological agents; for example, the DHFR inhibitors trimethoprim, pyrimethamine, and methotrexate (MTX) are widely used in the treatment of bacterial infections, malaria, and cancer, respectively.

Amplification of the DHFR gene with a proportional increase in protein has been shown to be a common mechanism of resistance in cultured tumor cells grown in the presence of increasing concentrations of MTX (1–7). In a majority of such cell lines, the overproduced enzyme is biochemically indistinguishable from the enzyme in cells which are sensitive to the drug. Several cell lines have been described, however, in which the amplified DHFR genes code for an enzyme with a reduced affinity for MTX. For example, Haber et al. (5) demonstrated that the DHFR in the highly MTX-resistant mouse cell line 3T6-R400 is amplified and altered, with a 240-fold reduction in MTX binding affinity. Subsequently, it was shown (8) that the altered properties of the enzyme in 3T6-R400 cells are due to an arginine substitution for the leucine residue 31. X-ray crystallographic studies (9) have shown that this leucine is located in the active site of the mouse DHFR and makes a hydrophobic interaction with bound MTX. It has also been established (10, 11) that amplified and altered DHFRs are associated with MTX resistance in several Chinese hamster lung cell lines. The first altered human DHFR to have its mutation identified at the molecular level was found and characterized in the MTX-resistant human colon adenocarcinoma cell line HCT 8R4 (12). Although the DHFR gene in HCT-8R4 cells is amplified only 25-fold, the cells can grow in the presence of MTX at a concentration that is 10,000-fold higher than the concentration which is tolerated by the parental cell line. Analysis of the nucleotide sequence from the DHFR cDNA from HCT-8R4 cells revealed that the phenylalanine at residue 31, which makes a hydrophobic interaction with bound ligands in the active site, had been mutated to a serine. Subsequent site-directed mutagenesis studies (13) demonstrated that this mutation lowers the affinity of the human DHFR for MTX by a factor of 100. In this study, we report the identification of a mutation in the DHFR gene from a MTX-resistant CHO cell line, Pro-3 MtxR™ (referred to as MtxR™), originally described by Flintoff et al. (14, 15). This cell line was developed by selection of cells expressing low levels of an altered DHFR with increasing concentrations of MTX; MtxR™ cells express higher levels of this altered enzyme. We have amplified in vitro both genomic DNA and transcripts of the DHFR gene from both the Pro-3 cell line (the parental line) and the MtxR™ subline using the polymerase chain reaction. Direct DNA sequencing of the amplified material revealed a phenylalanine substitution for leucine at residue 22.

EXPERIMENTAL PROCEDURES

Materials—Reagents for mRNA isolation and cDNA synthesis were obtained from Invitrogen (San Diego, CA). RNase-free siliconized tubes were obtained from National Scientific (San Rafael, CA). TaqI DNA polymerase was obtained from Perkin-Elmer/Cetus Instruments (Norwalk, CT) or Stratagene (San Diego, CA). Agarose was obtained from FMC (Rockland, ME). Centricon 100 microcentrifuges were obtained from Amicon Corp. (Danvers, MA). Reagents for DNA sequencing were obtained from United States Bio-

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1 The abbreviations used are: DHFR, dihydrofolate reductase; CHO, Chinese hamster ovary; MTX, methotrexate; PCR, polymerase chain reaction.
In Vitro DNA Amplification—Genomic DNA or DNA from first strand cDNA synthesis was used as template for amplification using the Thermus aquaticus (TuqI) heat-stable DNA polymerase as described previously (22,23). Generally, one-fifth of the cDNA reaction was performed in siliconized tubes in a 50-μl solution containing 50 mM potassium chloride, 10 mM Tris, pH 8.4, 2.5 mM magnesium chloride, 500 ng of each primer, a 200 PM concentration of each deoxyribonucleotide triphosphate, 25 mM magnesium chloride, and 10 units of reverse transcriptase were added, and the mixture was incubated at 42°C for 1 h.

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Synthetic Oligonucleotides—Oligonucleotides were synthesized by the solid-phase triester method in an Applied Biosystems Model 380 DNA synthesizer. Reaction products were cleaved and deprotected by standard protocols. Oligonucleotide primers for the PCR and DNA sequencing were similar to those described by Carothers et al. (21). Primers whose names end in B anneal to the 5'-region of the noncoding strand of the CHO DHFR gene, and primers whose names end in A anneal to the 3'-region of the coding strand. Primers for exon amplification are labeled EX, with the number corresponding to the respective exon. Primer CH03' was used for first strand cDNA synthesis in conjunction with CH05' for amplification. All primers are written from 5' + 3': EXlA, cgccaacttgcggagga; EX1B, ccgaagtctgcccgctt; EX2A, ctcttgagagttttctctctct; EX2B, ccccaagggagctcttctct; EX3A, caatggtatcttgctct; EX3B, agaataactcatagatctaa; CH03', gaacttgaagtcaatcagcaagtatctt; EX4A, atcagattacctgactagtat; EX4B, gataattggttctgctgctgct; EX5A, aatcttctagtttggctctctctcttctcttct; EX5B, cttcttgtaattctctcttct; EX6A, cttcttgtaattctctcttcttctctctcttctcttctct; EX6B, agataattctagactactgact; CH05', caactttggagga; and CH05', caactttggagga.

RESULTS

In Vitro Amplification and DNA Sequencing of DHFR Gene and Transcript—In vitro amplification of the entire peptide coding region of the CHO DHFR cDNA for both cell lines is shown in Fig. 1 (lower). The primers used for amplification of the cDNA anneal to the 5'- and 3'-untranslated regions of the transcript and do not overlap with the translated region. In addition, genomic DNA was specifically amplified for the six DHFR exons for both cell lines. The amplification product was verified by direct DNA sequence analysis. All TaqI DNA amplification reactions were run with controls to ensure that no contaminating templates were introduced from unintended sources.

Because of the greater ease in sequencing single-stranded DNA, we have taken advantage of the "asymmetric PCR technique" (27) after performing a conventional amplification reaction. Two mutations were found: a C → T transition at nucleotide 67, resulting in the substitution of phenylalanine for leucine at codon 22 (Fig. 1, upper; and Fig. 2), and a C → G transition at nucleotide 434, resulting in the substitution of serine for threonine. This was confirmed on the genomic level by amplifying and directly sequencing the respective exons (Fig. 3). Silent mutations were noted and are listed in Fig. 4.

Demonstration of Loss of BsaI Site in DHFR cDNA MtxHIII Cell Line—The mutation at nucleotide 67 causes a loss of the recognition site for the restriction enzyme BsaI. Genomic DNA from the Pro-3 or MtxHIII cell line was selectively amplified by the PCR to give a 600-base pair fragment (lanes 1 and 2). This was then digested with BsaI (an isochizomer of Ppal) (lanes 3 and 4); sequence recognition site is shown upper), which cuts the wild-type cDNA between nucleotides 62 and 67. The result of the C → T transition is the loss of the BsaI site in the MtxHIII cell line. Lane M contains 0.5 pg of phage X174 DNA digested with HaeIII used as a size marker. Lanes 1 and 3 represent the amplified product of the CHO Pro-3 DHFR cDNA; lanes 2 and 4 represent the amplified product obtained from the MtxHIII DHFR cDNA.
cDNAs. mRNA from cells was reverse-transcribed and amplified in vitro using symmetric and asymmetric PCRs. A single point mutation is seen at nucleotide 67 (C → T), resulting in the substitution of transition results in the loss of a BsaI site in MtxR1' cells, which was confirmed by restriction digests (see Fig. 1).

DNA from both cell lines was poorly digested by this enzyme, and Southern detection was not successful. In vitro amplified Pro-3 cDNA was efficiently cut with BsaI (Fig. 1, lane 3). The amplified 600-base pair fragment was cleaved by the restriction enzyme to give a faster migrating product. In contrast, the MtxR1' cDNA was not cut by BsaI (Fig. 1, lane 4).

Binding Kinetics for MtxR1' Cell Line—The MTX $K_i$ for the MtxR1' enzyme (0.19 nM) was determined by measuring the steady-state reaction velocity as a function of inhibitor concentration. The data were fit by nonlinear least-squares regression to equations for tight binding inhibitors which take into account the depletion of free inhibitor when the concentrations of inhibitor and enzyme are of the same magnitude (17). A value of 0.5 μM was determined for the dihydrofolate $K_i$ for the MtxR1' cell line (data not shown) and was used in the above fitting routine. Although an exact MTX $K_i$ for the parental Pro-3 CHO cell line has not been determined, MTX $K_i$ values for wild-type mammalian DHFRs, when determined in a manner similar to the one used in this study, have consistently been found to be in the single picomolar range (17, 28, 29). It is reasonable to conclude therefore that the MtxR1' DHFR binds MTX approximately 100-fold less tightly than does the wild-type enzyme.

**DISCUSSION**

In 1976 and 1980, Flintoff et al. (14, 15) described a series of CHO cell lines which were resistant to MTX. Studies with crude extracts from Class I cells suggested that resistance was due to a mutated DHFR with altered MTX binding properties. Class III cells were derived from Class I cells by another round of selection in a increased concentration of MTX and appeared to be relatively unchanged (data not shown). It is interesting to note that the $K_i$ for the MtxR1' DHFR phenylalanine 22 mutant (0.19 nM) is nearly identical to the $K_i$ values obtained for a human DHFR with a serine mutation at residue which have undergone stepwise selection in increasing concentrations of MTX (amplification of DHFR on the DNA, RNA, and protein levels), the level of resistance does not reflect the degree of DHFR amplification. Most cell lines which have exhibited a degree of MTX resistance similar to that of MtxR1' cells have been found to have a much greater level of gene amplification (100–200-fold), suggesting that MtxR1' cells are highly resistant to MTX by more than just gene amplification. Subsequently, studies were carried out on purified DHFRs from MTX-sensitive Class I and II resistant cells; these studies demonstrated that DHFRs from Class I and III cells were indeed identical and had a lowered affinity for MTX. We have also found that the MtxR1' DHFR has consistently been found to be in the single picomolar range (17, 28, 29). It is reasonable to conclude therefore that the MtxR1' DHFR binds MTX approximately 100-fold less tightly than does the wild-type enzyme.
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31 that is also associated with a MTX-resistant phenotype (12, 13).

In vitro amplification of the DHFR cDNA and exons and direct DNA sequencing confirmed that the high degree of MTX resistance exhibited by MtXX<sup>III</sup> cells was due to expression of an altered DHFR. Direct DNA sequencing of the DHFR gene from MtXX<sup>III</sup> cells on both the mRNA and genomic levels revealed two differences from the nucleotide sequence previously reported for the coding region of the CHO DHFR (11). These differences are the substitution of serine for threonine at residue 144 and the substitution of phenylalanine for leucine at residue 22. Several pieces of evidence suggest that the mutation at residue 22 is solely responsible for the altered properties of the DHFR in MtXX<sup>III</sup> cells. First, the serine mutation at residue 144 was also found in the DHFR gene of the parental MtX-sensitive Pro-3 cell line. Second, in the x-ray structures of all vertebrate DHFRs (30, 31) and mammalian cells (12, 13).

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REFERENCES

22. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi,
Altered DHFR Gene in the CHO Pro-3 MtX cell line

Identification and characterization of a mutation in the dihydrofolate reductase gene from the methotrexate-resistant Chinese hamster ovary cell line Pro-3 MtxRIII.
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