A STUDY OF RIBONUCLEOTIDE REDUCTASE FROM WILD-TYPE AND HYDROXYUREA RESISTANT MOUSE L CELLS IN CULTURE

by

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

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This thesis is dedicated to the unyielding love, tolerance, and understanding of my parents.

ABSTRACT

The antitumor agent hydroxyurea is cytotoxic to mouse L cells in culture. At a concentration of 0.35 mM, hydroxyurea stops cellular growth and reduces plating efficiency to 6 X 10^{-6} in wild-type cultures. Ribonucleotide reductase activity was partially purified from wild-type L cells, and the optimum assay conditions and kinetic characteristics were determined for the substrates CDP and GDP. Enzyme activity from wild-type L cells was found to be similar to enzyme activity isolated from other mammalian sources. In agreement with other studies, both CDP and GDP reductase activities were potently inhibited by hydroxyurea, suggesting that the cytotoxic effects of this drug are due to its inhibition of ribonucleotide reductase.

A somatic cell mutant was isolated which exhibits a pleiotropic phenotype rendering it resistant to normally highly cytotoxic concentrations of hydroxyurea and guanazole (3,5-diamino-1,2,4-triazole, NSC 1895). The drug resistant phenotype was stable during long periods of non-selective cultivation and appears to have arisen by mutational rather than epigenetic means. Ribonucleotide reductase activity was also studied in detail from partially purified extracts of the drug resistant cell line designated L_2Cl_3. The optimum assay conditions for CDP and GDP reduction in L_2Cl_3 extracts resembled the conditions employed with wild-type extracts. However, ribonucleotide reductase activity from L_2Cl_3 extracts exhibited some significant kinetic differences when compared with the wild-type enzyme. For example, the Km value for

CDP with the L_2Cl_3 enzyme preparations was 3-fold higher than the value for this substrate with the wild-type enzyme. Ribonucleotide reductase activity from L_2Cl_3 extracts was also less sensitive to inhibition by hydroxyurea, with both CDP and GDP reductase activities showing increased Ki values for hydroxyurea. These findings are consistant with the presence of a structurally altered ribonucleotide reductase enzyme in L_2Cl_3 cells.

A technique is described for measuring ribonucleotide reduction in small numbers of whole cells made permeable to nucleotides by treatment with the detergent Tween-80. This procedure produced high enzyme activity per cell and a linear response to enzyme concentration which did not occur in cell-free extracts. The in vivo measurement of CDP and GDP reductase activity also revealed a difference between wild-type and L_2Cl_3 cells in their response to assay temperature, which supports the presence of an altered enzyme in L_2Cl_3 cells.

The in vivo assay system was also used to measure levels of CDP and GDP reductase activity in nucleotide-permeable wild-type and L_2Cl_3 cells. It was discovered that L_2Cl_3 cells contained approximately 3 times the wild-type level of CDP reductase, and twice the wild-type level of GDP reductase activity. These enhanced enzyme levels complemented by a decreased sensitivity of the L_2Cl_3 enzyme to inhibition by hydroxyurea can account for the cellular resistance of L_2Cl_3 cells to the drug.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A genetic approach to the study of the cell biology of bacteria and lower eukaryotes has been instrumental to the understanding of many aspects of the biochemistry and molecular processes present in these organisms. A similar approach in the study of multicellular animals is severly hindered by their very complex phenotypes. However, with the development of techniques for cell culture, biologists are now able to cultivate pure lines of higher eukaryotic cells as if they were microorganisms. This has allowed mammalian cell biologists to complement biochemical studies with genetic studies in attempts to gain further insight into the nature of biochemical pathways and molecular controls in mammalian cells.

The field of somatic cell genetics has expanded enormously within the last decade, with much of the research involving the isolation and characterization of cultured somatic cells with variant phenotypes. The exact genetic nature of the hereditable variation of cells in culture has not often been established. However, many well characterized systems are now available in which the variation in phenotype can be attributed to mutational rather than epigenetic events.

The work reported in this thesis involves the isolation and characterization of a variant mouse L cell line which is resistant to the cytotoxic effects of the anti-tumor agent hydroxyurea. This study involves the determination of the nature of the hydroxyurea resistant phenotype in this variant of an aneuploid permanent cell line.

Evidence exists indicating that hydroxyurea exerts its cytotoxic effects through inhibition of ribonucleotide reductase, an enzyme which plays a very important role in the synthesis of DNA. Therefore, ribonucleotide reductase activity was partially purified and characterized from extracts of wild-type and hydroxyurea resistant L cells. Optimal assay conditions and kinetic characteristics of the enzyme were compared to determine if the drug resistant cell line contained a ribonucleotide reductase enzyme which was structurally altered from the wild-type enzyme.

The study of ribonucleotide reductase activity in cell-free extracts presents a number of difficult problems. In an attempt to minimize these problems, a method was developed to measure enzyme activity in small numbers of whole cells made permeable to nucleotides by treatment with the detergent Tween-80. This procedure allowed the measurement of *in vivo* levels of ribonucleotide reductase in mouse cells. Ribonucleotide reductase levels were compared in wild-type and drug resistant L cells to determine if enhanced enzyme levels played a role in resistance to hydroxyurea.

HISTORY

MUTANTS OF SOMATIC CELLS IN CULTURE

The complete understanding of cellular processes in any living ststem is dependent upon our ability to understand the genetic basis of these functions. For many years, the study of basic cell biology in bacteria has been complemented by the isolation and characterization of mutant cells (for review see Harris, 1964). Such cells, which can show a variety of alterations or defects in phenotype, have contributed greatly to the elucidation of biochemical pathways and molecular controls.

Attempts have been made to apply the same methodology of genetic analysis used with bacteria to similar studies in multicellular organisms (eg. Simmons and Crow, 1977; Ward, 1977). These studies are severely hindered, however, by the immense complexity of the phenotypes of these organisms.

The development of cell culture techniques was an enormous breakthrough in allowing a genetic approach to studying the biology of multicellular organisms. Pure lines of mammalian cells that would grow in semi-defined medium provided cell biologists with a valuable tool for qualitative and quantitative analysis of genetic and biochemical properties of the multicellular animal. It

is quite natural that a great deal of the recent work being done on somatic cells in culture has revolved around the selection, isolation, and characterization of cells with stable alterations in their phenotype.

Clements (1975) has emphasized the importance of considering cultured mammalian cells as a population of individuals capable of expressing subtle differences not readily detectable under standard growth conditions. It is this background variation which produced the first reported variant cell lines in culture. Puck and Fischer (1956) observed that cloning of a HeLa cell population would isolate variant cell lines with altered growth requirements. A short time later, the first drug-resistant variants were isolated in culture; among a variety of cell types, variants were isolated with resistance to folic acid antagonists (Vogt, 1959; Szybalski, 1959), antipurines (Lieberman and Ove, 1959), and antipyrimidines (Morris and Fischer, 1960).

Since that time, the field of somatic cell genetics has expanded rapidly. Many reviews have been published (eg. Clements, 1975; Siminovitch, 1976; Basilico, 1977; Wasmuth and Caskey, 1978), all of which emphasize the immense variety of mutant phenotypes which have been isolated from somatic cells in culture.

Perhaps the major controversy in the study of somatic cell genetics has to do with the origin of the observed

genetic variations of cells in culture. Stable alterations in cellular phenotype may arise at different levels of organization in the cell, and hence may have a genetic or epigenetic origin (Harris, 1971). A true genetic mutation has been defined as a genetically based alteration in phenotype which has resulted from any hereditable change in the DNA; this includes changes in primary structure due to deletions, point mutations, etc., as well as chromosome rearrangement and/or loss (DeMars, 1974; Siminovitch, 1976). Epigenetic changes may also arise. These have been described (Nanney, 1968) as being the result of directed shifts in phenotypic expression which do not involve alterations in the DNA per se, but nevertheless result in hereditable variations.

Epigenetic changes on a programmed basis are a familiar part of embryonic development and cellular differentiation (Coon, 1966, Gehring, 1968). Similarily, Harris (1971) has suggested that permanent cell lines which are grown in a highly artificial environment may exhibit unstable mechanisms of genetic expression; and may exhibit stable phenotypic changes during cell culture which are brought about by epigenetic mechanisms rather than actual mutational events. It has been suggested, in fact, that most variant somatic cells in culture have arisen by epigenetic means (Harris 1971,1973,1975; Mezger-Freed 1971, 1972). The support for this statement comes from many different

investigators who provide evidence contraindicating a mutational basis to a variety of observed variant phenotypes. For example, many variant phenotypes are reported to be unstable when grown for long periods under non-selective conditions (Littlefield, 1965; Morrow,1970; Mezger-Freed, 1971; Alt et al, 1978). Also, cultured somatic cells had often shown mutation rates which were much higher than the accepted rates in bacteria, and hence were thought to arise by mechanisms other than gene mutation (Breslow and Goldsby, 1969; Harris,1971, 1973; Mezger-Freed, 1972). In addition, the mutation rates in these cultured somatic cells did not always increase when the cells were exposed to agents known to be mutagenic to bacterial cultures (Mezger-Freed, 1972, Szybalski et al, 1964). Examples were also uncovered where the frequency of variants observed did not appear to depend on cell ploidy. Harris (1971), for instance, examined the frequency of occurence of variant Chinese Hamster cells which were resistant to a purine analogue or thermal shock. It was observed that the rates of occurence of these markers was approximately the same in cultures of diploid, tetraploid, or octaploid Hamster cells; and therefore was not in support of the assumption that the variant phenotype was the result of a dominant, co-dominant, or recessive change at the gene level. This was supported by Mezger-Freed (1972) who observed that the rate of occurrence of

bromodeoxyuridine-resistant frog embryo cells did not appear to depend on cell ploidy, as it should if the alteration in phenotype was due to a gene mutation.

A controversy exists, however, and many of the observations of Harris and Mezger-Freed have been severely challenged by DeMars (1974), who also points out that mutation rates for drug resistance in cultured somatic cells are in accordance with rough estimates of germinal mutation rates in humans. A great deal of data has since accumulated to support a genetic basis of hereditable mutation in cultured somatic cells. The enhancing effect of mutagens in increasing the frequency of occurence of variants in many genetic systems is now widely recognized (see reviews by Chu, 1974; Siminovitch, 1976) and has been carefully quantitated (eg. Hsie et al, 1975a, 1975b; Friedrich and Coffino, 1977). Also, many investigators have shown that mutation rates in cultured somatic cells respond to cell ploidy in a manner consistant with a mutational basis for the genetic variation (eg. Chasin, 1972, 1973; Chasin and Urlaub, 1975; McBurney and Whitmore, 1974; Jones and Sargeant, 1974; Raskind and Gartley, 1978).

It seems certain that many isolated variants have a stably altered phenotype due to an actual alteration in their DNA. Such variants would therefore be classed as true mutants. The ultimate evidence indicating the presence of a true mutation, however, would involve sequenc-

ing the DNA of the wild-type parent and observing alterations in the mutant. Failing this, somatic cell geneticists must rely on evidence which will infer that an observed variation is mutationally based. Towards this end, a number of criteria have been established which, ideally, should all be satisfied when defining a mutant somatic cell line. The major requirements include (Chu, 1974; Clements, 1975):

1. Spontaneous random occurence at a low frequency
2. Retention of stable phenotype in the absence of selection
3. Increased frequency of appearance of mutants after exposure to mutagens
4. Production of an altered gene product
5. Interallelic complementation
6. Localization of the lesion on a specific chromosome
7. Expression in somatic hybrids in a recessive, dominant, or co-dominant fashion

Following is a discussion of some of the better documented examples of hereditable variations of apparent mutational origin.

A. Drug Resistance

Drug resistance is perhaps the most studied mutant phenotype of somatic cells in culture. Recent reviews on the subject (Clements, 1975; Siminovitch, 1976; Wasmuth and Caskey, 1978) exemplify the wide variety of drug resistant mutants available. Also, Clements (1975) has summarized the many adaptations which alone or in combination may result in drug resistance in cultured somatic cells.

Purine analogue resistance: The purine analogues 6-thioguanine, 8-azahypoxanthine, 8-azaguanine, and 6-mercaptopurine are toxic to mammalian cells after being processed by the enzyme hypoxanthineguanine phosphoribosyltransferase (HPRT), an enzyme which is non-essential to the cell under certain culture conditions. These analogues have therefore been used to select for cultured somatic cells deficient or lacking HPRT enzyme activity (phenotype termed $hprt^-$) (eg. Lieberman and Ove, 1959; Littlefield, 1963; Fujimoto et al, 1971, DeMars, 1974). The resistant phenotype is stable for long periods in the absence of drug and the frequency of mutation is increased by mutagenesis (Chu and Malling, 1968; Hsie et al, 1975). These mutants also appear phenotypically identical to cells obtained from human males with the Lesch-Nyan syndrome where the defect has been located on the X chromosome (Seegmiller et al, 1967; Fujimoto et al, 1971)

There is a great deal of evidence for the production of an altered gene product in $hprt^-$ mutants. Cell lines with the $hprt^-$ phenotype have been isolated which produce an enzymatically inactive protein which cross reacts immunologically (CRM^+) with antibodies to purified HPRT enzyme (Beudet et al, 1973; Wahl et al, 1975). Recently, Capecchi et al (1977) reported the presence of a CRM^+ protein in $hprt^-$ L cells which was found to have an altered carboxy-terminal peptide. Chromatographic analysis

strongly suggested that the mutant peptide was shorter than the HPRT wild-type peptide. It was then postulated that the hprt$^-$ phenotype resulted from a nonsense mutation in the HPRT gene. This was strongly supported by the finding that the mutant could be "phenotypically corrected" by microinjection of bacterial or yeast ochre-suppressor tRNA, but not wild-type tRNA. It was then claimed that this mouse L cell hprt$^-$ variant was a genetically based suppressible nonsense mutant.

Resistance to purine analogues via loss of HPRT activity may, of course, arise by means other than nonsense nutation. For example, Fenwick et al (1977) reported the isolation of Chinese hamster hprt$^-$ variants with an altered enzyme of the same molecular weight as the wild-type enzyme, but with different substrate binding properties. It was suggested that the hprt$^-$ phenotype was the result of a missense mutation in the HPRT gene. Further evidence for the production of an altered enzyme in hprt$^-$ variants has been obtained from a Chinese Hamster Ovary (CHO) cell mutant which shows temperature-sensitivity for both HPRT activity and purine analogue resistance (Fenwick and Caskey, 1975).

In conclusion, it appears that at least some of the variant cells resistant to purine analogues satisfy most of the major criteria for classification as a genetically based mutant cell line. The altered phenotype is stable;

its occurence is increased by mutagenesis, and the lesion has been localized on the X chromosome. In addition, the evidence of the production of an altered gene product is strong.

Hydroxyurea resistance: Stable resistance to the cytotoxic effects of the drug hydroxyurea can be obtained from CHO cells (Wright and Lewis, 1974; Lewis and Wright, 1978b). The variant phenotype is stable during extended cultivation under non-selective conditions, and its occurence in the wild-type population is enhanced by mutagenesis (Lewis and Wright, 1978b). Hydroxyurea-resistant cell lines have been isolated which produce an altered target enzyme, ribonucleotide reductase (Lewis and Wright, 1978a). The altered enzyme showed a several fold higher resistance to hydroxyurea in vitro when compared to the wild-type enzyme. Other variants isolated owed their resistance to the production of enhanced levels of drug-sensitive ribonucleotide reductase (Lewis and Wright, 1978c; Lewis, Kuzik, and Wright, 1978).

α-amanitin resistance: α-amanitin inhibits the enzyme RNA polymerase II , and is therefore cytotoxic to cultured mammalian cells. Many cell lines have been isolated, however, which are resistant to normally toxic levels of α-amanitin. For example, α-amanitin-resistant CHO cells have been isolated which produce an altered RNA polymerase II enzyme, which can account for the drug resistant pheno-

type (Lobban et al, 1976; Ingles et al, 1976).

Emetine resistance: Gupta and Siminovitch (1976) have isolated mutant CHO cells which are resistant to the protein synthesis inhibitor emetine. Drug resistance in these cells has been attributed to the production of an altered 60S ribosomal subunit which is less sensitive to inhibition by emetine than is the wild-type 60S subunit ((Gupta and Siminovitch, 1978).

cAMP resistance: Daniels et al (1973) reported that S49 mouse lymphoma cells would lyse in the presence of elevated levels of cAMP in the culture medium, but variants could also be isolated which were cAMP-resistant. It has since been shown that many cAMP-resistant phenotypes are the result of a mutational event which has produced an altered regulatory subunit of a cAMP protein kinase enzyme (Hochman et al, 1975; Steinberg et al, 1977).

Oubain resistance: Oubain is normally cytotoxic to cultured mammalian cells via its inhibition of Na^+/K^+ ATPase (Baker et al, 1974). Oubain-resistant variants have been isolated, however, and appear to have resulted from a mutational event which has led to the production of an altered Na^+/K^+ ATPase enzyme which is resistant to inhibition by oubain (Baker et al, 1974, Robbins and Baker 1977)