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ISOLATION OF A HUMAN LYMPHOBLASTOID LINE HETEROZYGOUS AT THE THYMIDINE KINASE LOCUS: POSSIBILITY FOR A RAPID HUMAN CELL MUTATION ASSAY

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SUMMARY: A thymidine kinase heterozygote designated H2BT has been isolated from the human lymphoblast line HH4. Significant increase in the trifluorothymidine-resistant fraction was observed in the new cell line following treatment with the mutagens ICR-191 and butylmethansulfonate. Phenotypic expression was complete forty-eight hours after treatment.

INTRODUCTION

Clive and Flamm have developed a selection system for forward mutation in L_{5178Y} mouse lymphoma cells using the thymidine analog bromodeoxyuridine (BUdR) as a selective agent (1,2,3). Resistance to BUdR results from the mutational loss of thymidine kinase (TK; EC2.7.1.21), an enzyme which phosphorylates thymidine and its analogs in an ATP-dependent reaction (2). Once phosphorylated, BUdR is further metabolized and eventually incorporated into DNA, where it is believed to exert its toxic effect. Another thymidine analog, trifluorothymidine (TFTdR), which was developed by Heidelberger (4,5,6) can also be used in the TK selection system.

An interesting feature of the TK selection system is its extremely short phenotypic expression period of approximately 48 hours (2). This phenomenon is probably due to the cell-cycle-dependent appearance and disappearance of TK in the cell (7,8,9,10).

Our laboratory has recently developed a human lymphoblast mutation assay using 6-thioguanine resistance as a genetic marker (11). We felt it advantageous to include the TK selection system in this assay for two reasons. First,

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the expression time for TFTdR resistance should be much shorter than the extremely long expression time (~10-14 generations) required for 6-thioguanine resistance (11,12), thus making experiments less cumbersome. Secondly, the tk locus would provide a second genetic marker to allow comparison of the mutation frequency observed at the hgprt locus in human cells.

Since the tk locus is located on autosome 17 in humans (13,14), we first had to construct a $TK^{+/-}$ heterozygote to allow use of the TFTdR selection system. Here we present the selection scheme used to isolate the $TK^{+/-}$ line and present preliminary mutation data using ICR-191 and butylmethanesulfonate.

MATERIALS AND METHODS

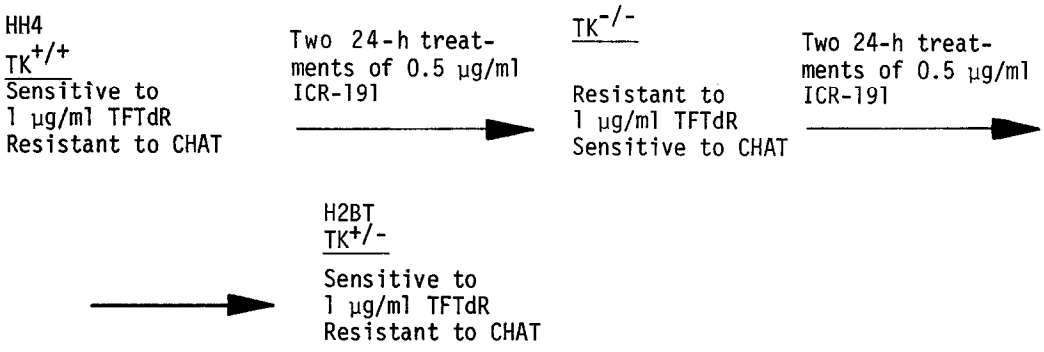
Chemicals: Chemicals were obtained from the following sources: trifluorothymidine, thymidine, cytidine, aminopterin, α-glycerophosphate, and adenosine triphosphate from Sigma Chemical Company, St. Louis, MO; [^{3}H]-thymidine and Aquasol from New England Nuclear, Boston, MA; butylmethanesulfonate from Pfaltz and Bauer, Inc., Stamford, CT; hypoxanthine from Nutritional Biochemical Corp., Cleveland, OH; ICR-191 from Dr. H.J. Creech of The Cancer Institute, Fox Chase, PA; and Agarose (ME) from Marine Colloids, Inc., distributed by Miles Laboratories, Inc., Elkhart, IN.

Lymphoblastoid Line: The HH4 lymphoblastoid line used in these studies was derived from the near diploid WI-L2 line, which was originally isolated from a male spherocytosis patient. Unlike other diploid lymphoblast lines, HH4 can form macroscopic colonies in soft agar in the absence of a feeder layer of human fibroblasts. With these cells, we were able to circumvent the arduous task of isolating a TFTdR-resistant human fibroblast line.

Medium: RPMI 1640 medium, supplemented with 15% fetal calf serum (Flow Laboratories, Rockville, MD), was used to grow all lines. CHAT medium consisted of RPMI supplemented with 1×10^{-5}M cytidine, 2×10^{-4}M hypoxanthine, 2×10^{-7}M aminopterin, and 1.75×10^{-5}M thymidine. After treatment in CHAT, cells were grown for one day in RPMI containing only hypoxanthine, cytidine, and thymidine to reverse the aminopterin inhibition.

TK Enzyme Assay: A modification of the method developed by Furlong (15) was used. Approximately 10^6 cells were washed in saline and resuspended in 1 ml of 0.1 M Tris-HCl with 2.5 mM mercaptoethanol. The cell suspension was sonicated with four 15-second bursts and then centrifuged at 9000 g for 30 minutes at 0°C. The supernatant (cell sap) was then decanted. Each reaction mixture contained the following: 40 µl cell sap, 40 µl 0.1 M Tris-HCl buffer (pH 8.0), and 20 µl H_2O containing 0.005 µmoles [^{3}H]-TdR (200 mCi/m mole), 0.5 µmoles ATP, 0.6 µmoles α-glycerophosphate, and 0.5 µmoles $MgCl_2$. The reaction was run in plastic conical 15 ml centrifuge tubes (Falcon Plastics, Oxnard, CA). The tubes were incubated at 37°C for 30 minutes, and then immersed in boiling water for 2 minutes to terminate the reaction. The tubes were then centrifuged at 2000 rpm for 15 minutes to precipitate the denatured protein. The supernatants were decanted and 20 µl samples were taken and spotted on Whatman DE81 filter papers. The filters were washed in a chimney apparatus with 20 ml 5 mM NH_4HCO_3 followed by 20 ml ethanol. The filters were then dried and added to 10 ml Aquasol and counted.

Isolation of $TK^{+/-}$ Heterozygote: The following scheme, analogous to the one described by Clive (2), was used to isolate $TK^{+/-}$ strain H2BT:



The potent frameshift mutagen ICR-191 was used to enrich for mutants because it causes high frequencies of non-leaky mutations which can be reverted by additional treatment with ICR-191 (16). Twenty-four hour exposures to 0.5 µg/ml ICR-191 were used, since this treatment gives maximum mutation with minimum toxicity to the lymphoblasts (17).

In the initial step to find the $TK^{-/-}$ homozygote, approximately 2×10^8 $TK^{+/+}$ HH4 cells ($\sim 5 \times 10^5$/ml) were twice treated with ICR-191 in stationary culture. Four days after the last treatment 1 µg/ml TFTdR was added to the culture which was then left at 37°C for 3 days to further enrich for mutants. The culture was then plated in the presence of 1 µg/ml TFTdR in RPMI supplemented with 20% FCS and solidified with 0.25% agarose. Ten TFTdR-resistant clones were isolated and purified. To determine if the mutants isolated were actually $TK^{-/-}$, the TK enzyme assay described above was performed. Each mutant possessed approximately 5% of the activity of the parent $TK^{+/+}$ population. This residual activity is probably due to mitochondrial TK released by sonication(18,19).

To isolate the $TK^{+/-}$ heterozygote we selected one of the $TK^{-/-}$ clones and twice treated approximately 10^8 cells with ICR-191. Three days after the final treatment, the culture was plated in CHAT medium supplemented with 20% FCS and solidified with 0.25% agarose.

Nine CHAT-resistant clones were isolated. The mutants were tested for TK enzyme activity and found to have only 25-50% of the wild-type ($TK^{+/+}$) level. The heterozygous nature of one of these mutants, designated H2BT, was further tested in the mutation assay described below. We expected that the observed mutant fractions to TFTdR resistance (spontaneous and induced) in a TK heterozygote would be several orders of magnitude greater than for the $TK^{+/+}$ described above.

Mutation Assay: H2BT was maintained in spinner culture at 37°C with a doubling time of approximately 18 hours. Approximately 10^7 cells (4×10^5/ml) were treated with either BMS (1 mM) or ICR-191 (0.25 µg/ml and 0.50 µg/ml) for 24 hours. After treatment, the cells were centrifuged and resuspended in fresh medium at a concentration of 3×10^5/ml. Dilutions to 3×10^5/ml were made daily.

At various times after treatment, duplicate aliquots of 4×10^6 cells were centrifuged and resuspended in 10 ml of RPMI containing 20% FCS, 100 u/ml penicillin, and 0.25% agarose (plating agar). To determine the plating efficiency of the culture, a 10 µl sample of the suspension containing 4000 cells was added to 5 ml of plating agar and layered over 5 ml prejelled layer of the same medium in a 100 mm petri dish. A 10 µl aliquot of 2 mg/ml solution of TFTdR was added to the 10 ml suspension. The suspension was then plated in two 5 ml aliquots, each layered over a prejelled layer of plating agar in a 100 mm petri dish (final TFTdR concentration = 1 µg/ml). The plates were fed the following day with 3 ml of medium.

The plates were counted after 7-10 days in a 37°C, 5% CO_2, humidified incubator.

Results from the mutation assay with BMS and ICR-191 are presented in Table I.

TABLE I. Induction of TFTdR-resistant Mutants in H2BT Following Treatment with ICR-191 and BMS

Compound and Dose	Days Post-Treatment	Observed Mutants per 4×10^6 Cells	Plating Efficiency	Estimated Mutant Fraction $\times 10^6$
ICR-191	0	26	0.25	26
(0.25 µg/ml x 24 Hrs)	2	139	0.26	130
	4	126	0.27	120
ICR-191	0	55	0.24	57
(0.5 µg/ml x 24 Hrs)	2	229	0.25	230
	4	182	0.20	230
BMS	1	132	0.094	350
(1 mM x 24 Hrs)	3	275	0.15	460
	5	387	0.23	410
0 CONTROL	1	1	0.23	1.1
	3	1	0.22	1.1
	5	3	0.35	2.1

DISCUSSION

The lymphoblast line HH4 is presumably a $TK^{+/+}$ homozygote. This is supported by the observation that treatment with ICR-191, a potent mutagen for human cells (6), resulted in a TFTdR-resistant fraction of less than 10^{-7} (the exact fraction cannot be determined due to a three-day allowance for growth in suspension culture). We showed that the TFTdR-resistant, CHAT-sensitive cells isolated were $TK^{-/-}$ homozygotes by their deficiency of TK enzyme activity. The subsequent CHAT-resistant cells which were recovered at a frequency of about 10^{-7} had a TK enzyme activity of 25-50% of HH4; this is consistent with the hypothesis that they are indeed $TK^{+/-}$ heterozygotes.

The fraction of TFTdR-resistant mutants in the $TK^{+/-}$ H2BT line was significantly increased after treatment with ICR-191 and BMS. Since the appropriate

reconstruction experiments testing for bias are still in progress, we cannot at this time quantitate with certainty the amount of mutation induced and we therefore have labelled the mutant fractions presented in Table I as estimates. However, we are assured that the qualitative increase in mutant fraction is real, since the greater than 100-fold increase in the number of mutants, which occurred only a few days after treatment, could not have resulted from the selection of pre-existing mutants. The ICR-191-induced TFTdR-resistant fraction of H2BT is approximately 10^{-4}, which is at least 1000-fold higher than the fraction observed for a more intensive treatment of HH4. This further supports our belief that H2BT is a $TK^{+/-}$ heterozygote, while HH4 is a $TK^{+/+}$ homozygote. We also note that the estimated TFTdR-resistant mutant fraction induced by ICR-191 and BMS are comparable in magnitude to the 6-thioguanine-resistant fraction induced by these compounds (17,20).

It is our belief that the use of human cells in in vitro screening assays best reflects the human in vivo situation because of the important species differences in DNA/chromatin structure, DNA repair, membrane structure, and metabolism. In addition to being theoretically sound, any assay method must also be practical in terms of cost and time required to allow large-scale application. One of the operational drawbacks of the human cell assay based on 6-thioguanine resistance was the extremely long time which cells must be cultured to allow phenotypic expression (2). However, the use of the TFTdR selection system developed by Clive and coworkers (1,2,3) has helped overcome this problem and has made mutation experiments in human cells much more facile.

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