DoE/ER/60502-3

DOE/ER/60502--03

DE89 013800

THE DEVELOPMENT OF IN VITRO MUTAGENICITY TESTING SYSTEMS USING T-LYMPHOCYTES

RESEARCH PROGRESS REPORT: June 1, 1988 - May 31, 1989

(Budget Period November 1, 1988 - October 31, 1989)

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May 1989

Prepared for

The U.S. Department of Energy

Grant No. DE-FG02-87ER60502

VI. Progress Report and Background for Future Studies

A. Overview

This section will describe our progress during the past 3 years in quantifying the frequencies of mutations in human T-lymphocytes and analyzing the molecular bases of these mutations. It will be divided into three sub-sections.

The first sub-section will summarize our refinement of the methodology for cloning human T-lymphocytes and quantifying in vivo T-cell mutant frequencies at the hprt locus. The ability to maintain in vitro proliferation of these cells allows molecular characterizations, including analyses of hprt gene changes and T-cell receptor (TCR) gene rearrangement patterns. As outlined elsewhere, the latter permits definitions of mutation frequencies which underlie the measured mutant frequencies. This unique aspect of the T-lymphocyte cloning assay is extremely important because it permits quantitation of in vivo mutational events, which are the events of public health interest. An increase in in vivo mutational events is only one of the causes of increased mutant frequencies, the other is in vivo clonal expansions of mutant cells. Clonal expansions have an importance of their own, but the implications are different than for increases in mutations. Several examples of in vivo clonal expansions will be detailed.

The ability to maintain in vitro proliferation of T-cell colonies also allows molecular analyses of the mutations in the hprt gene. The results of both Southern blot analyses for gene deletions and rearrangements and direct sequencing for smaller changes such as base substitutions will be given. A spectrum of hprt mutations is being developed both for unexposed, "normal" humans as well as for humans exposed to ionizing irradiation in the course of radioimmunoglobulin therapy (RIT) for hepatoma treatment.

The DOE has been the secondary source of support for our in vivo studies in that it supported the methodological developments necessary for efficient in vitro growth of human T-lymphocytes to numbers sufficient for molecular analyses, and for a portion of the analyses themselves. Also, the in vivo studies of RIT patients were initiated with support from this DOE grant. These studies ultimately achieved their own support from the NCI.

The second sub-section will summarize our development of an assay to quantify the in vitro induction of hprt mutations in human T-lymphocytes. This development required adaptation of our methods for in vitro proliferation of T-cells to obtain optimal proliferation and subsequent cloning from mass cultures of T-lymphocytes maintained with interleukin-2 (IL-2). Gamma irradiation of G_0 phase cultures was employed as the mutagenic treatment. Phenotypic expression time and optimal selection cell density have been defined. Dose response relationships for normal humans are reproducible, permitting the assay to be used on larger population samples. Mutants induced by 300 rad external beam gamma irradiation have been analyzed by Southern blots employing both TCR and hprt gene probes. The TCR gene rearrangement patterns are used to define the independent nature of induced mutations and the hprt analyses to establish the spectrum of deletions and rearrangements

responsible for these mutations. Recent improvements in the assay include a method for limiting-dilution expression of these mutations to eliminate the complication of overgrowth of "sibling mutants" and to increase the efficiency of molecular analyses. The reproducibility of the in vitro assay allows its use for studying the heterogeneity in the human population of susceptibility to mutagens, and for defining single agent in vitro hprt mutational spectra. The DOE has been the sole source of support for all of our in vitro studies.

The third sub-section will describe our application of measures of DNA repair to human T-lymphocytes. This sub-section is provided as background for the current grant application. None of these studies have thus far been supported from DOE sponsors. Measures of DNA repair provide a second measure of mutagen sensitivity in the human population. In addition, correlations between repair capacity, cytotoxicity and mutation induction can now be investigated in the same cells from any individual.

B. Specific areas of progress

1. Studies of hprt mutations occurring in vivo in human T-lymphocytes.

a. Human T-cell hprt mutant frequencies

In order to validate the reproducibility of the in vivo T-cell hprt clonal assay, we undertook a longitudinal study of 6 individuals (3 male, 3 female) over a six-month period. The average mutant frequency was $5.4 \pm 2.4 \times 10^{-6}$ which is consistent with our earlier studies of 115 assays ($6.5 \pm 4.8 \times 10^{-6}$) (Albertini et al., 1988). The mutant frequencies for each individual were constant over time and over experimenter and showed slight differences among individuals. These data are detailed in the preprint "Longitudinal study of the in vivo mutant frequency in human T-lymphocytes as determined by a cell cloning assay" by J.P. O'Neill, L.M. Sullivan, J.K. Booker, B.S. Pornelos, M.T. Falta, C.J. Greene, and R.J. Albertini, Environmental and Molecular Mutagenesis (in press; appendix).

b. Use of T-cell receptor (TCR) gene rearrangement patterns to define independence of in vivo hprt mutants, and in vivo differentiation stage of the mutations.

Each T-cell has a specific antigen receptor on its cell surface. Usually this consists of an $\alpha\beta$ protein dimer although some T-cells have a $\gamma\delta$ dimer. There proteins are created by rearrangements of the T-cell receptor (TCR) genes (α, β,; γ, δ) during T-cell differentiation in the thymus. A unique set of rearrangements marks a T-cell and its clonal descendants. Southern blotting of the hprt mutant T-cell clones with TCR gene probes allows detection of these gene rearrangements and ascertainment of whether two or more clones share a set of rearrangements, identifying them as clonal descendants of the same in vivo progenitor (Nicklas et al., 1986, 1987). If, in addition, these mutants show the same alteration at hprt they are "sibling" mutants. The hprt mutants that are siblings do not result from independent mutations and must be corrected for in calculations and analyses of mutational events. Therefore, the independence of T-cell mutant isolates must be known

in order to make definitive statements regarding mutant frequencies or mutational spectra (e.g. "mutational hotspots").

TCR gene analyses also allows the determination of the differentiation stage in vivo of the hprt mutations (i.e. pre- or post-thymic). Mutants with different hprt alterations but the same TCR gene rearrangement patterns occurred independently in post-thymic cells, i.e. after TCR gene patterns had been fixed. By contrast, sibling pre-thymic mutants and those independent post-thymic mutants that result from hprt mutations in mutational "hotspots" will show another characteristic pattern when groups of such mutants are analyzed, i.e. the same hprt alteration but different TCR gene rearrangements. These two can be distinguished however, because independent post-thymic "hotspot" mutants will show the same hprt change in many individuals, while sibling pre-thymic mutants will not.

Southern blot analysis using TCR gene probes of 326 hprt mutants and 94 wild type clones from the 3 male individuals (D, E, and F) in the longitudinal study described above, showed that approximately 90% of the hprt mutants had different TCR gene rearrangements and thus represented independent mutations. All of the wild type T-cell isolates were independent in that they originated from different in vivo progenitors, indicating that the hprt mutants derived from a somewhat restricted in vivo sub-population. Most of the TCR gene sharing in the 10% of hprt mutants that showed sharing was the result of doublets (i.e. 2 isolates with the same TCR gene rearrangement pattern), although triplets, quadruplets and one nonamer were seen. The average persistence of a set of TCR gene identical hprt mutant clones in the 3 individuals was 2.8, 2.9 and 1.0 months, respectively. Clones of the nonamer were obtained over a period of 10 months and, in fact, this clone persists at about the same level (10% of hprt mutant isolates) after 2 years. These results are detailed in "Molecular analyses of in vivo hprt mutations in human T-lymphocytes III. Longitudinal study of hprt gene structural alterations and T-cell clonal origins" by J.A. Nicklas, T.C. Hunter, J.P. O'Neill and R.J. Albertini which has been submitted to Mutation Research (in appendix).

c. In vivo clonal expansions of hprt mutant T-cells in humans

We have studied in detail a "normal" woman with a very high hprt T-cell mutant frequency (~85×10^{-6} on 2/84 to ~700×10^{-6} at present) (Nicklas et al., 1988). This high frequency is due entirely to the expansion of one T-cell clone as defined by TCR gene rearrangement analysis. In actuality, this woman's mutation frequency is not increased although her measured mutant frequency is. Karyotypic analysis has shown that some of the hprt mutants included in the in vivo clonal expansion have undergone loss of one X-chromosome, one has pericentric inversion of chromosome 7 and one has a variable number of chromosomes. Sequencing studies of the hprt mutation suggests that a single hprt event occurred early in the expansion of this clone in vivo (possible exon 6 "splice site" mutation), but that secondary events may have occurred in some of the isolates. This suggests that the hprt mutations are occurring in the dividing cells, perhaps as a consequence of division. Furthermore, some wild type T-cell isolates from this individual now show the same TCR gene rearrangement pattern, originally recognized only among the mutants, suggesting that the overall expanding clone is much larger

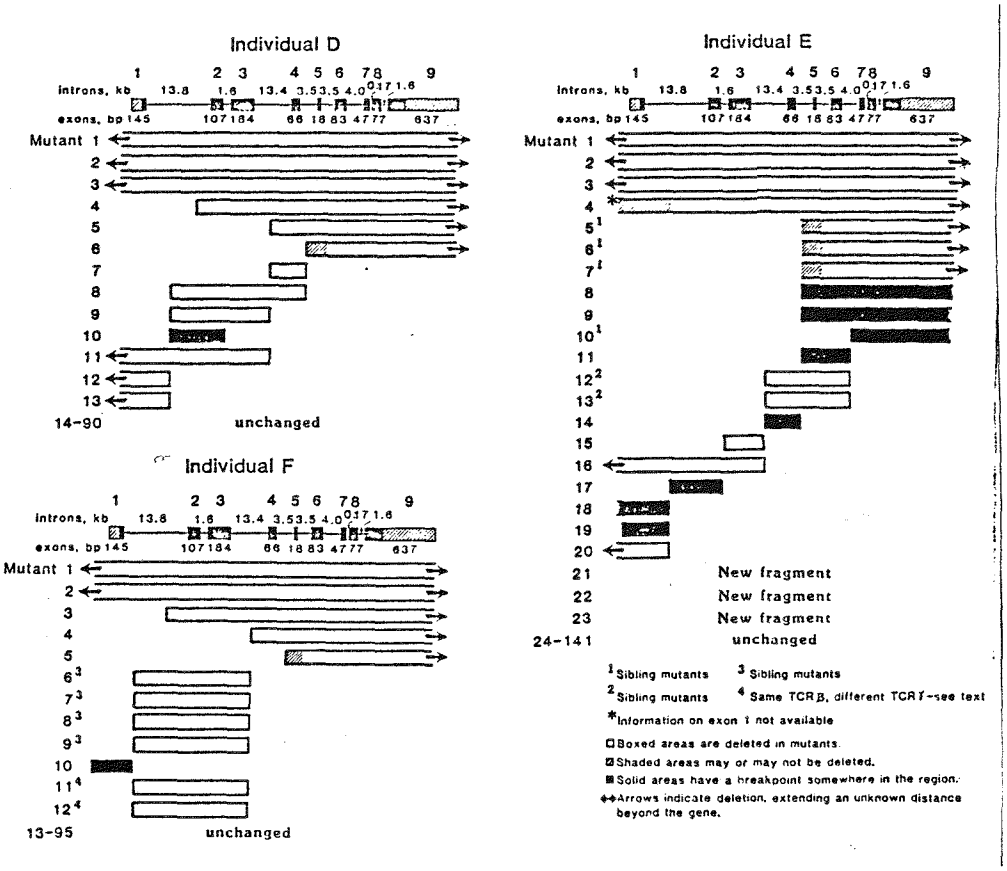
than the hprt mutant sector, and that the hprt mutation(s) occurred after the clonal amplification began.

This individual (and her cells) are under continued study in our laboratory because of the possibility that we have identified "pre-malignant" changes in this clone. Other examples of T-cell clonal amplification have been identified in other individuals. We are currently starting several studies that use the hprt assay with TCR gene analyses to detect in vivo T-cell clonal expansion. In this scheme, hprt mutation serves merely to signal in vivo cell division, with the hypothesis being that "spontaneous" hprt (or any other) mutations occur preferentially (or exclusively) in the small subset of T-cells in vivo that are undergoing active or recent cell division.

d. hprt mutational spectrum defined by Southern blots.

Molecular analyses of the 326 hprt mutant clones from the 3 male individuals showed that 14% of the mutants had gross hprt gene structural alterations on Southern blots. These results are discussed in detail in the manuscript cited above. There were a wide variety of alterations (see Figure 1); the breakpoints of which were spaced evenly across the gene. Based on the frequency of breakpoints per kb and the number of internal hprt versus external breakpoints, a minimum size of deletable region was calculated to be approximately 90kb.

FIGURE 1 - Mutants from Individuals D, E and F



We have begun sequencing studies of the 86% of mutants which do not show hprt alterations on Southern blots to obtain a complete spectrum of "spontaneous" hprt mutation. These studies are being performed in collaboration with Dr. Thomas Skopek at Chemical Industry Institute of Toxicology, Research Triangle Park, NC. Analyses is being performed by polymerase chain reaction (PCR) of cDNA, cloning and then sequencing using one M13 and one internal hprt primer on an Applied Biosystems 370A automatic DNA sequencer. The changes obtained in the first 20 mutants from individual E are shown in Table 1.

TABLE 1 - Sequences of Individual E Mutants

Mutant	cDNA Change[1]	Amino Acid[2]	Comments
JB67 E10B3	$T_{623}\rightarrow A$	$ile_{207}\rightarrow asn$	
MF223 B4D4	$T_{623}\rightarrow A$	$ile_{207}\rightarrow asn$	
JB67 D10G2	$A_{404}\rightarrow G$	$asp_{134}\rightarrow gly$	
JB67 E10A5	$C_{482}\rightarrow GG$	$ala_{160}\rightarrow gly$	frameshift to new term at aa_{173} (TAA at 174)
JB67 D3E7	$G_{400}\rightarrow A$	$glu_{133}\rightarrow lys$	
JB67 E9B1	$G_{601}\rightarrow T$	$asp_{200}\rightarrow term$	
JB67 D10D9	$G_{212}\rightarrow A$	$gly_{70}\rightarrow asp$	
BP31 A4F11	$G_{569}\rightarrow A$, $A_{570}\rightarrow T$	$gly_{189}\rightarrow asp$	
BP31 A4C5	$G_{599}\rightarrow A$	$arg_{199}\rightarrow lys$	
JB67 E8H4	$T_{\text{intron 5 base 1}}\rightarrow G$		addition of 65 bp to new apparent alt splice site in intron 5, new aa's from glu_{133} to new term at aa_{141}
JB67 B9H11	Δ A_{80} to T_{94}	loss his_{26}-asp_{31}	inframe loss of 5 amino acids
JB67 C7A12	Δ $T_{583}A_{584}$ or $A_{584}T_{585}$ or $T_{585}A_{586}$	$tyr_{194}\rightarrow term$	2 bp deletion creates termination codon
JB67 D4A2	Δ exon 8		splice site weakened (?), some mRNAs OK
MF223 D5E7	Δ exon 8		splice site weakened (?), some mRNAs OK
JB67 E10G8	Δ exon 5		Δ, splice site mut (?)

JB67 D10F2	Δ exon 8	Δ, splice site mut (?)
BP26 A6D1	Δ exon 4	Δ, splice site mut (?)
BP26 A9F10	Δ exon 7	Δ, splice site mut (?)
BP12 A4A1	Δ exon 7	Δ, splice site mut (?)
MF223 E7G11	addition of the 3′ 14bp of intron 8 between the exon 8 and 9 sequences	new splice acceptor site 14 bp 5′ into intron 8 (?), frameshift and 36 aa longer protein

[1]Alterations seen in cDNA sequence, numbering starts at 1 for A of AUG start codon
[2]Numbering starts with 1 with ala after first met

There are a variety of changes including base pair changes, multiple base losses, exon losses (probable splice site alterations) and one partial inclusion of an intron (also a probable splice site mutation). We have also sequenced a number of mutants from the woman with the high mutant frequency (Table 2).

TABLE 2 - Sequence of hprt Genes in Clones of a Woman with High Mutant Frequency ($>600 \times 10^{-6}$)

Mutant	cDNA change	Amino acid	Comments
M4	Δ exon 6		
M11	Δ exon 6		
M13	$C_{113} \rightarrow T$	$pro_{37} \rightarrow leu$	
M14	Δ exon 6		
M21	Δ exon 6		
M26	Δ exon 6		
M31	Δ exon 6		
M35	Δ exon 6		
M49	Δ exon 6		
M59	Δ exon 6		
M61	Δ exon 6		
M63	Δ exon 6		
M78	Δ exon 6		
M92	Δ exon 6		
M203	Δ exon 6		
M207	Δ exon 6		
M211	Δ exon 6		
M223	Δ exon 6, Δ exon 8		
M236	Δ exon 6		
M249	Δ exon 6, +T after G_{96}, +T after G_{129}	$glu_{55} \rightarrow$term	first T →immediate term
M277	Δ exon 6		

M284	Δ exon 6
M291	Δ exon 6, Δ exon 8
M295	Δ exon 6

[1]Alterations seen in cDNA sequence, numbering starts at 1 for A of AUG start codon
[2]Numbering starts with 1 with ala after first met

As expected most show the same cDNA alteration (a deletion of exon 6) but several mutants show additional changes as well, indicating secondary hprt mutations upon the first. This implies that mutation is occurring in this dividing clone. The one mutant showing only a base pair mutation is an independent, non-sibling mutant that is not part of the expanding clone and thus should show a unique hprt alteration.

We are beginning studies to map the extent along the X-chromosome of deletions with breakpoints external to the hprt gene. We have obtained a collection of 19 probes mapping to Xq26-27 within about 10cM of the hprt gene (Table 3).

TABLE 3 - hprt linked probes

DX#	probe	ATCC - donor	insert size	enzyme	vector	drug resist	reference
DXS10	36B-2	57062	1.4kb	Hind	puc9	amp	Som Cell Mol Ge 10:607
DXS79	07-03	57373	2.4kb	EcoRI	puc19	amp	Cyto Cell Gen 46:665
DXS107	cpX234	57276	.15kb	BamHI	puc12	amp	Am J Hum Gen 39:438
DXS92	pXG-16	57148	4.3kb	HindIII	pAT153	amp	NAR 13:7540
DXS51	p52a	57172	5.3kb	EcoRI/ HindIII	pBR322	amp	Science 230:753
DXS102	cX38.1	57288	2.6	EcoRI	pAT153	amp/tet	Am J Hum Gen 39:438
DXS177	plambda 2.7	57405	7kb	EcoRI	puc19	amp	HGM8
DNF1	pAX-6	57182	1.44kb	PstI	pAT153	tet	Som Cell Mol Ge 10:385
DXS100d	pX45d	White	0.5kb	HindIII	puc8	amp	Cyto Cell Gen 37:486

DXS100h	pX45h	White	2.5kb	HindIII	puc8	amp	NAR 14:5572
DXS99	pX58dII Ic	White	2.4kb	HindIII	puc8	amp	Hum Genet 75:38
DXS19	pX46d	White	1.8kb	HindIII	puc8	amp	Cyto Cell Gen 42:123-128
DXS37	30RIb	White	6.28kb	EcoRI	pAT153	amp	Cyto Cell Gen 42:123-128
DXS144	C11	Mandel	1.0kb	PstI	pEMBL8	amp	Hum Gen 77:60
DXS86	ST1	Mandel	6.0kb	EcoRI	pBR329	amp/tet ?	Hum Gen 77:60
DXS172	pX71c	White	???	HindIII	puc8?	amp	Cyto Cell Genet 42:123
DXS173	pX76a	White	???	HindIII ?	puc8?	amp	Cyto Cell Genet 42:123
DXS174	pX82d	White	???	HindIII ?	puc8?	amp	Cyto Cell Genet 42:123
DXS59	L2.9	Bakker	???	EcoRI?	pBR322?	amp?	HGM7

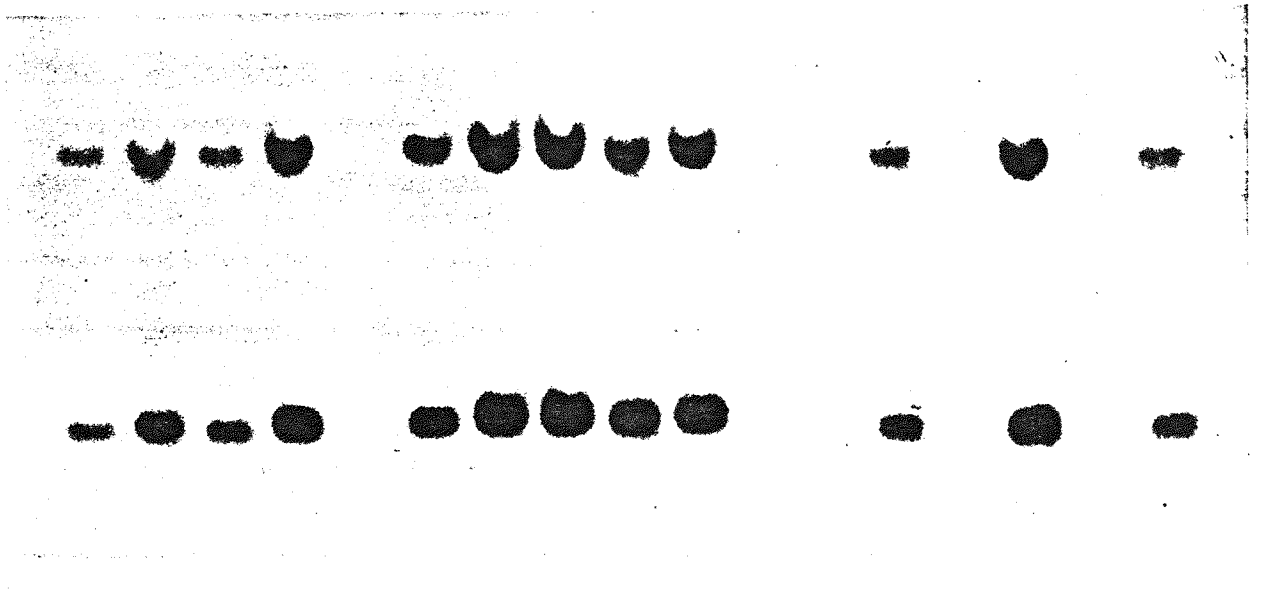
Using regular Southern blotting we are checking if any of the loci detected by these probes are deleted in the hprt mutants. A Southern blot using the probes DXS10 and DXS144 of 4 wild type, 5 total hprt deletions, and 3 partial hprt deletions is shown in Figure 2.

Note that 2 of the total deletions have lost the DXS10 locus as well as the hprt gene. (Estimates of the genetic distance between hprt and DXS10 have ranged up to 14cM, indicating that rather large deletions of the human X-chromosome can possibly be recovered as viable T-cell hprt mutants.) We plan to develop a map of the hprt region using the different probes and a set of deletions. Once we have found the most closely linked markers, we plan to use pulsed field gel electrophoresis (which separates large DNA pieces) to estimate the size of deletions by shifts in the sizes of the fragments of linked but undeleted markers.

FIGURE 2 - PstI Southern blots using hprt gene linked probes

A) probe DXS10

B) probe DXS144



LANE	1	2	3	4	5	6	7	8	9	10	11	12
	wild type				total deletions					exon 1	exons5-9	exons4-9
										partial gene deletions		

e. Studies of hprt T-cell mutations in newborns and aged twins.

We have also undertaken a study of in vivo T-cell hprt mutations in newborns and aged twins. The mean frequency for 45 normal newborns was 0.64 x 10^{-6} which is approximately 10 fold lower than normal adults. These results are detailed in "In vivo hprt mutant frequencies in T-cells of normal Human newborns" by M.J. McGinniss, M.T. Falta, L.M. Sullivan and R.J. Albertini Mutation Research (in press). Molecular analysis of 41 hprt mutant clones showed that major hprt gene alterations occurred in 85% of the mutations with most (~90%) having breakpoints in introns 1 and 3. This is a much different mutational spectrum than is seen in adults where ~15% of the mutants have hprt gene alterations and the breakpoints are evenly spread across the gene (in adults, only 63% of the breakpoints are in introns 1 and 3). Also, the lack of external breakpoints suggests the close proximity of vital genes in the fetus, which is also dramatically different from findings in the adult (see above). The other difference between newborn and adult mutations are in the frequencies of prethymic hprt mutations. Seven instances of sets of mutants with the same hprt mutation but different TCR gene rearrangements were found indicating that hprt mutation in the fetus occurred in stem cells before thymic differentiation (and TCR gene rearrangement). Only 2 such instances have been found in all the adult clones studied. These results are detailed in "Molecular analyses of in vivo hprt mutations in human T-lymphocytes IV. Studies in newborns" by M.M. McGinniss, J.A. Nicklas and R.J. Albertini which has been submitted to Environ. and Molec. Mutagenesis. We are beginning a larger study of newborn mutants in collaboration with Dr. David Manchester at Children's Hospital in Denver.

The aged twins are being studied in collaboration with Dr. Betsy Hirsch at the Univ. of Minnesota. The average mutant frequency for 36 twin pairs is 17.7 ($\pm$ 14.3) x 10^{-6} which is slightly higher than for young adults. Molecular studies of these mutants are in progress.

f. Studies of in vivo hprt mutation in Down Syndrome patients.

In vivo mutant frequency values, using the autoradiographic assay, for 8 Down syndrome patients of various ages showed a mean of 20.9 X 10^{-6}, $\pm$ 11.5 while similar aged, normal concurrent control subjects had a mean mutant frequency value of 8.7 X 10^{-6}. The control values are consistent with our other studies of normals, while the Down Syndrome results are elevated.

g. In vivo hprt T-cell mutation in patients receiving total body irradiation via radio-immunoglobulin therapy (RIT).

We are studying humans who have been exposed to total body ionizing irradiation. Patients with certain forms of cancer have shown benefit from treatments with radioimmunoglobulin therapy (RIT). This consists of intravenous administration of polyclonal anti-ferritin antibodies to which radionuclides have been covalently attached. The anti-ferritin antibodies localize to tumors, but also to bone marrow and other reticuloendothelial tissues in vivo. This delivers total body irradiation from internal emitters. Patients undergoing RIT receive substantial irradiation, often necessitating autologous bone marrow transplantation. In addition to RIT, patients receive

preliminary external beam irradiation to the tumor, and concomitant low dose chemotherapy. We currently have a paper in preparation "Molecular analyses of *in vivo* hprt mutations in human T-lymphocytes V. Effects of total body irradiation secondary to radioimmunoglobulin therapy (RIT)" by R.J. Albertini, M.T. Falta, J.A. Nicklas, J.P. O'Neill, D. Jacobson-Kram, and J.R. Williams which will be submitted shortly to *Environmental and Molecular Mutagenesis*.

Twenty seven assays were performed on blood samples from 23 patients treated with RIT containing ^{131}I. These patients, the doses received, the time interval from treatment to test, and Mf values are given in Table 4.

TABLE 4

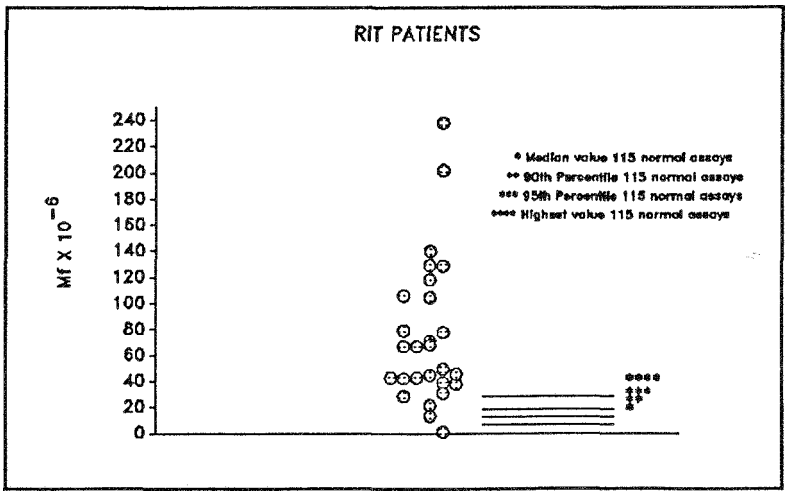
Patient	Age[1]	Dose in mCi		in vivo Expression Time[2]	CF[3]	MF[4]
		Last	Cumulative			
SC	27	46	46	2 months	0.10	42.5
		51	97	2 months	0.03	37.6
		50	147	2.5 months	0.03	201.6
		50	147	7 months	0.04	66.9
WQ	20	29	150	2 months	0.03	139.7
		27	177	2 months	0.02	128.5
HK[5]	49	30	30	2 months	0.05	42.6
		30	30	2 months	0.17	44.5
RK	35	29	284	2 months	0.07	71.2
		30	314	2 months	0.04	68.0
TT	80	30	30	4 months	0.07	38.7
RBr	72	30	90	2 months	0.03	104.4
PMc	42	51	51	2 months	0.02	222.7
EH	83	30	260	2 months	0.02	235.5
RR	51	31	31	2 months	0.12	13.1
BR	40	31	31	2 months	0.05	49.2
JT	72	30	131	2 months	0.43	28.4
RBa[6]	45	30	30	2 months	0.08	118.2
RS	43	30	60	2 months	0.15	77.5
RM[6]	25	30	30	2 months	0.23	45.3
GF	62	50	100	2 months	0.06	79.1

EG	77	30	60	6 months	0.03	22.5
BN	54	30	60	2 months	0.01	128.9
JM	41	28	178	2 months	0.04	66.9
MC	28	29	208	2 months	0.20	21.3
UH	27	49	49	2 months	0.08	235.7
JC	61	29	29	2 months	0.02	105.8

[1]Age in years at time of treatment
[2]Time interval from last RIT to test
[3]CE = cloning efficiency = - ln P_0 / inoculum size
[4]Calculated mutant frequency = - ln P_0 (Selection Wells) /inoculum size / CE
[5]Replicate blood samples obtained 7 days apart: same patient, same treatment
[6]Received ~5mCi ^{111}In localizing dose

As can be seen, the patients ranged in age from 20 to 83 years. RIT was administered at 2 month intervals, unless held for toxicity. Total cumulative doses of ^{131}I ranged from 29 to 314 mCi, while the last dose received by individual patients ranged from 27 to 51 mCi. The mean Mf value for these 27 assays is 90.2 (SD = 66.7) x 10^{-6} (range = 13.1 - 235.7 x 10^{-6}). The median Mf value for these 27 assays is 68.0 x 10^{-6} (10th and 90th percentiles are 29.7 and 231.7 x 10^{-6} respectively) These values are shown graphically and compared to "background" Mf values previously reported for normal young adults in Figure 3.

FIGURE 3 -

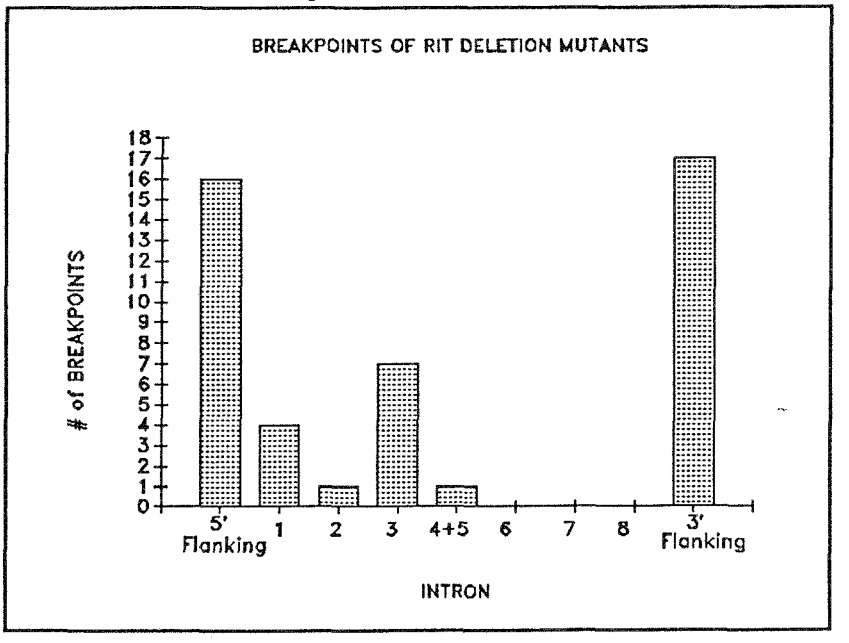


Although Mf values for patients receiving RIT are clearly much higher than those previously found for normal adults (Figure 3), it was not possible to show a dose-response relationship for the current study. Among the patients receiving ^{131}I RIT, linear regression analyses showed no significant correlation of Mf value with age of patient ($r = 0.0699$), with total cumulative dose (mCi) of ^{131}I received ($r = 0.1501$), or with the amount of ^{131}I (or Ci) received in the last dose, ($r = 0.3244$). However, in those nine instances where clonal assay was performed 2 months after the first RIT ^{131}I injection, there was a significant correlation of Mf value with dose ($r = 0.6944$; $p. < 0.05$), although the dose range was small (29-51 mCi).

A total of 23 wild type and 104 hprt mutant T-cell clones, isolated from 10 patients in 12 clonal assays, were studied by Southern blot analysis for hprt structural alterations and TCR gene rearrangement patterns. Thirty-six of the 104 mutant isolates showed hprt structural alterations that were either large enough to be detected by Southern blot analysis, or involved a restriction site change. When corrected for replicate isolates of sibling mutants, these 104 mutants could conservatively be attributed to 96 in vivo hprt mutational events, of which 32 involved large hprt changes. Therefore, 35% of the mutants, and 33% of the mutations involved gross changes in the hprt gene.

The breakpoints of the simple deletions and more complex hprt rearrangements were analyzed as to intron or extra-genic region where they most likely occurred. Results of this analysis, shown in Figure 4, indicate that breakpoints occurred linearly along the length of the gene, with no predilection for site other than size of the intron.

FIGURE 4 - Distribution of Breakpoints in RIT mutants



Based on this, breaks secondary to RIT occurred at a frequency of 0.0043 breaks/kb/mutation. (This compared to frequency of 0.0025 breaks/kb/mutation for "spontaneous" hprt breakpoints reported earlier for normal young adults.) Also, since 72% of the breakpoints occurred outside of the 43kb gene, with almost equal occurrences 5' and 3' to the gene, it suggests that a deletion of approximately 154 Kb can occur at this X-chromosomal site and still yield a viable mutant. This differs from the normal unirradiated adult where only 52% of the breakpoints occurred outside the gene giving a viable deletion size of 90kb.

h. Other in vivo studies

Several other human population studies of T-cell hprt mutations in vivo are ongoing. These include longitudinal studies of Breast Cancer patients, patients receiving platinum-containing chemotherapies, various auto-immune patient groups, and some occupationally exposed individuals. We therefore have a large cumulative experience with in vivo T-cell mutagenicity assays, and a large material for in vivo - in vitro comparisons.

2. Studies of mutations induced in vitro in human T-lymphocytes.

In order to analyze the mutations responsible for the in vivo derived mutant T-lymphocytes, methods were developed to maintain proliferation of cells in vitro as presented in the previous section. These growth conditions were then applied to allow in vitro proliferation and cloning of mass cultures in order to study the in vitro induction of mutation in these cells.

a. Induction, expression and selection of hprt mutants in T-lymphocyte G_0 phase cultures exposed to ionizing radiation.

We have defined the conditions which allow in vitro proliferation of human T-lymphocyte cultures and subsequent cloning of these cells. These conditions are detailed in the attached reprint and submitted (O'Neill, J.P., Nicklas, J.A., Hunter, T.C., Sullivan, L.M. and R.J. Albertini. Molecular Analysis of Mutations at the hgprt Locus in Human T-lymphocytes, 1987 and O'Neill, J.P., Sullivan, L.M. and Albertini, R.J. In vitro Induction, Expression and Selection of Thioguanine Resistant Mutants with Human T-lymphocytes. Mutation Res. (submitted)). These defined conditions include medium, optimal amount of T-cell growth factor (TCGF) or interleukin-2 (IL-2), irradiated lymphoblastoid feeder cell density and T-lymphocyte subculture regimen. These parameters have also been defined for optimal single cell cloning. Gamma irradiation of G_0 phase peripheral blood T-lymphocytes was then employed and cytotoxicity and mutation induction determined. For the mutation induction measurement, the time required for expressions of the hprt mutant phenotype was defined with cultures maintained in exponential growth. Cell cloning was measured in the absence and presence of 6-thioguanine to quantify the hprt mutant frequency. In addition, the optimal selection cell density was determined. Based on these results, a procedure has been defined for quantitative measurement of mutation induction by gamma irradiation. This includes mutant selection after 7-8 days of culture for phenotypic expression and a selection cell density of 1×10^4 cells per well.

This methodology does allow description of a dose response for both cytotoxicity and mutagenicity with gamma irradiation exposed cultures. However, these studies of in vitro induced mutation have been limited by the absolute requirement of T-lymphocyte proliferation and cloning on TCGF or IL-2. Batches of crude TCGF prepared by us are generally small relative to the amounts needed for these in vitro studies. Commercially prepared TCGF is costly and proved to be quite variable in usefulness for the cloning of in vitro grow T-cells (although it was generally suitable for the in vivo assay). Recently, we have found that the IL-2 containing medium employed for a lymphokine activated killer (LAK) cell procedure for cancer treatment is an excellent source of growth factor for this in vitro mutation induction assay. We have now defined conditions for its use as described below. This advancement will greatly facilitate these in vitro studies.

b. Molecular analysis of hprt mutations induced in vitro by ionizing irradiation.

The ability to measure mutation induction in vitro in cultures exposed to short duration external beam gamma irradiation also allows us to investigate the molecular basis for these mutations. This spectrum of mutations can then be compared with the spectrum observed in mutants arising in vivo in humans exposed to total body ionizing irradiation such as the RIT patients described earlier in this progress report.

Mutants were isolated from cultures exposed to 300 rad of external beam gamma irradiation and expanded for DNA studies. Because of the need for 5-7 days of bulk culture for phenotypic expression, sibling progeny of each induced mutation would be expected. We took advantage of the TCR gene rearrangement patterns in order to distinguish sibling colonies and thus define the independent nature of a mutation. The DNA alteration responsible for each mutation was analyzed with the hprt gene probe. These results are given in detail in the attached reprint and submitted manuscript (O'Neill, J.P., Nicklas, J.A., Hunter, T.C., Sullivan, L.M. and R.J. Albertini. Molecular Analysis of Mutations at the hgprt Locus in Human T-lymphocytes, 1987 and O'Neill, J.P., Hunter, T.C., Sullivan, L.M., Nicklas, J.A. and Albertini, R.J. Southern Blot Analyses of Human T-lymphocyte Mutants Induced in vitro by Gamma Irradiation. Mutation Res. (submitted)).

A summary of the results obtained with independent irradiations of T-lymphocytes from a single individual is shown in Table 5. The use of the TCR gene probes allows us to define the independent nature of mutations by differentiating sibling mutants. Many progeny of a single mutation are often isolated in the group of colonies grown for analysis. Alternatively, mutants with the same alteration can be shown to be independent events by use of the TCR gene probes. In this group of mutants induced by 300 rads, deletions and rearrangements of the hprt gene predominate (18/24 = 0.67) and the deduced breakpoints within the gene appear to be randomly distributed. As was observed in the RIT patients, total deletions were frequently found.

TABLE 5 Molecular nature of hprt mutations induced in vitro by gamma irradiation (300 rads)

Exp. no. (Mutant Freq.)	Mutants/ Mutations	hprt changes[a] BamHI	Hind III
P 35			
(32×10^{-6})	29 mutants 6 mutations		
	14 - exon 2-3 MW change	-22,+33kb	-7,+11kb
	3 - exon 2-3 deletion	-22kb	-7kb
	5 - exon 5-9 MW change	-	-17,+30kb
	1 - exon 2-9 deletion	-22,-12,-3.7kb	-
	5 - no change	NC	-
	1 - no change	NC	-
LS 148			
(91×10^{-6})	19 mutants 8 mutations		
	4 - exon 2-9 deletion	-22,-12,-3.7kb	-17,-7,-4.9kb
	2 - exon 2-9 deletion	-22,-12,-3.7kb	-17,-7,-4.9kb
	1 - exon 2-9 deletion	-22,-12,-3.7kb	-17,-7,-4.9kb
	1 - exon 2-9 deletion	-22,-12,-3.7kb	-17,-7,-4.9kb
	4 - exon 2-3 deletion	-22kb	-
	1 - exon 6-9 deletion	-12kb	-
	4 - no change	NC	NC
	2 - no change	NC	NC
LS 155			
(24×10^{-6})	17 mutants 2 mutations		
	8 - exon 2-9 deletion	-22,-12,-3.7kb	-17,-7,-4.9kb
	9 - exon 2-3 MW change	-22,+7kb	-7,+12kb
LS 38			
(64×10^{-6})	5 mutants 4 mutations		
	2 - exon 2-3 MW change	-22,+25kb	NC
	1 - exon 4-9 deletion	-12,-3.7kb	-17,-4.9kb
	1 - exon 5-9 MW change	-12,+30kb	-17,+12kb
	1 - no change	NC	NC

P 40

(47×10^{-6})	4 mutants		
	4 mutations		
	1 - exon 2-3 MW change	-22,+24kb	NC
	1 - exon 5-9 MW change	NC	-17,+15kb
	1 - exon 5-9 MW change	-12,+6,+10kb	-17,+20kb
	1 - no change	NC	NC

[a] The changes in hprt restriction fragment patterns are listed. NC means no change was observed; - means that digest was not performed.

The isolation of sibling progeny can be defined by this method of analysis and so independent mutations can be clearly distinguished. However, the analysis is clearly inefficient since 70 mutant colonies represented only 24 independent mutations. We have recently developed a limiting dilution method of expression which allows the isolation of essentially 100% independent mutations. The essential element in this improvement was our characterization of the use of the LAK supernatant as a source of IL-2, as described in the next section.

c. Use of lymphokine activated killer cell (LAK) therapy supernatant for the in vitro proliferation of human T-lymphocytes.

The in vitro proliferation and single cell cloning of T-lymphocytes is uniquely dependent on exogenous TCGF or IL-2 for growth stimulation. This dependence has necessitated considerable effort in characterizing TCGF activity and optimal amount for use in this assay. We have employed batches of crude TCGF prepared in our laboratory as well as obtained commercial sources. All showed the same characteristics in that most batches which showed activity in stimulating the proliferation of mass cultures of human T-lymphocytes could also be employed for cell cloning of PHA activated T-cells in both the presence and absence of TG. However, only some of these batches could be used for the in vitro expansion of these isolated T-cell colonies to cell numbers sufficient for molecular analyses of the mutations. A variety of experiments suggested that the amount of PHA in these preparations was an important aspect in defining the usefulness of crude TCGF preparations. In our hands use of partially purified TCGF with the PHA removed or recombinant IL-2 did not allow optimal T-cell cloning or efficient expansion of the isolated colonies. During the course of these studies, a clinical procedure for cancer therapy was initiated at the University of Vermont. This lymphokine activated killer cell (LAK) therapy employs incubation of the patients mononuclear cells at 1-3 x 10^6 cells/ml in medium containing 1500-3000 units/ml of recombinant IL-2 for 3-4 days. The cells are then recovered for infusion back into the patient and the supernatant medium discarded. Because of indications that this medium contains the exogenous IL-2 as well as IL-1 produced by the cells, we decided to investigate the use of it for T-cell cloning. The absence of PHA seemed to be a potential advantage

because we then had the ability to empirically define the optimal amount of PHA for the in vitro growth of isolated colonies.

The initial experiments employed peripheral T-lymphocytes activated by incubation with 1 ug/ml PHA for 36-40h. Cloning medium employed irradiated lymphoblastoid feeder cells at 5 x 10^3-1 x 10^4 cells per well. These cells showed optimal cloning both in the presence and absence of TG in medium containing 10-20% LAK supernatant (equivalent to 150-600 units/ml of IL-2 based on the amount originally added for the LAK therapy) and 0-0.5 ug/ml PHA. The addition of 1 ug/ml PHA to the cloning medium greatly reduced the cloning efficiencies in the absence of TG (1-5 cells/200ul) Optimal cell cloning was defined by the results obtained with our most active batch of crude TCGF and ranged from 80-90% based on the fraction of MNC which were T-cells (by a pan T-cell monoclonal antibody). However, we were unable to expand isolated T-cell colonies in medium containing 10-20% LAK supernatant and less than 0.1 ug/ml PHA. (Colony expansion was always performed with irradiated lymphoblastoid feeder cells at 2.5 x 10^5 cells per cm^2) Optimal conditions for colony expansion were found to be medium containing 0.1-0.25 ug/ml PHA. These conditions have allowed the routine expansion of 70-90% of isolated colonies to cell numbers of 15-20 x 10^6, our target number for Southern blot analysis. In addition, subsequent growth of cryopreserved cell samples to target cell numbers of 60-100 x 10^6 for hprt cDNA sequencing have been 90% successful.

The next set of experiments was designed to define the optimal conditions for the in vitro growth of mass cultures of T-lymphocytes, a necessary step for the study of in vitro induction expression and selection of hprt mutations. (All growth of mass cultures employed irradiated lymphoblastoid feeder cells at 2.5 x 10^5 cells per cm^2.) Cells activated by incubation for 36-40h. with 1 ug/ml PHA showed identical growth characteristics in medium containing at least 20% LAK supernatant and 0-0.5 ug/ml PHA for the first 11-14 days of in vitro culture. With longer growth time, 0.125-0.25 ug/ml PHA seemed to yield better proliferation, with slower growth at both less (down to 0) and more (0.5-1 ug/ml) PHA. The optimal cell proliferation conditions yielded population doubling times of 18-22h over the studied interval of 21-25 days of in vitro growth. Cells were generally subcultured at 1 x 10^5 cells/ml at 3 day intervals. Despite this active proliferation, these mass cultures showed a range of cloning efficiencies (in the absence of TG) when cell cloning was performed with the same amount of PHA used for proliferation (0, 0.125, 0.25, 0.5, and 1.0 ug/ml, respectively), with maximal cloning efficiencies found in the presence of 0.125 or 0.25 ug/ml PHA. Subsequent studies with cells grown for 11 days in vitro and then plated for cloning showed similar mass culture growth characteristics with all amounts of PHA (0-1.0 ug/ml) but clearly the best cloning efficiencies for cell cultures were found at 0.125-0.25 ug/ml PHA. Parallel studies with irradiated cells (300 rads) showed the same pattern for cloning efficiencies (in the absence of TG) i.e. equivalent with all amounts of PHA. However, TG^r mutant selection showed an unusual pattern in which cultures grown with 0 ug/ml PHA showed the highest mutant fraction with a 50% decline at a PHA amount of 0.3125 ug/ml. These strange results have been repeated with blood samples from 5 individuals. While we have no definitive explanation, we have

defined apparently optimal conditions for the use of LAK supernatant for studies of the in vitro induction of hprt mutations. This procedure now employs irradiation subculture for 8 days in 20% LAK supernatant containing 0 ug/ml PHA and cell cloning in the presence and absence of TG in medium containing 0.125 ug/ml PHA. Under these growth and selection conditions, fairly reproducible results could be obtained in approximately 50% of the determinations. The difficulty appears to be in the cloning step.

To define the best conditions for cloning of in vitro grown cells, we investigated every constituent of the cell cloning medium. These studies showed the irradiated feeder cells to be the crucial constituent for cell cloning. Addition of a feeding step 4 days after plating the cells greatly increased the TG^r cell cloning and is now routinely employed in the assay. Typical results for different feeding media are given below. Unirradiated cultures of G_0 phase cells and cultures exposed to 300 rads of gamma irradiation were subcultured for 8 days. They were then plated in 200μl at 1 and 2 cells/well in the absence of TG (CE) and at 1 x 10^4 cells/well in the presence of TG. Four days later the wells were fed with 50 μl of medium which consisted of RPMI 1640 containing 20% HL-1, 5% FBS, 50% LAK, and 0.125 ug/ml PHA, and the following:

	Control		300 rads	
	CE	$TG^r(10^{-6})$	CE	$TG^r(10^{-6})$
Experiment 1				
1) none	0.15	7.0	0.16	23.0
2) + 2 x 10^5 irradiated feeders/ml	0.33	5.8	0.23	96.0
Experiment 2				
1) none	0.18	5.8	0.16	17.0
2) + 2 x 10^5 irradiated feeders/ml	0.28	7.4	0.21	117.6

The feeding greatly increased the growth of TG^r clones and is totally due to the presence of irradiated feeders. Other combinations of growth factor, PHA, etc. had no effect. In addition, use of more irradiated feeder cells at the time of plating did not increase the TG^r frequency. The addition of more feeders 4-5 days after plating seemed optimal. Employing this cloning methodology, we have defined the phenotypic expansion time for hprt mutants. Examples of 3 determinations are:

	Mutant frequency (x 10^{-6}) at day				
	2	5	8	11	14
Individual A					
0 rads	8.3	5.3	3.7	6.9	5.4
300 rads	13.3	49.7	66.0	78.8	67.2
Individual B					
0 rads	4.6	5.2	6.4	2.1	
300 rads	9.2	98.9	83.7	57.3	
Individual C					
0 rads	6.6	4.5	3.2	1.7	2.4
300 rads	8.5	89.8	168.8	170.3	147.2

Employing an expression time of 8 days, dose response determinations for irradiated G_0 phase cells yielded the following results:

	Mutant frequency (x 10^{-6}) at radiation dose (in rads) of					
	0	50	100	200	300	400
Individual A	12.2	14.5	29.2	17.8	84.4	165.7

These studies of irradiation induced mutation are proceeding with other individuals in order to define the dosimetry of mutations induced by gamma irradiation in G_0 phase human T-lymphocytes.

This characterization of the use of LAK supernatant for T-lymphocyte culture led to a second improvement in the in vitro mutation assay. As described above in this progress section and in the attached manuscript (O'Neill, J.P., Hunter, T.C., Sullivan, L.M., Nicklas, J.A, and Albertini, R.J. Southern Blot Analyses of Human T-lymphocyte Mutants Induced in vitro by Gamma Irradiation. Mutation Res. (submitted)). The basic nature of in vitro mutation induction studies with the need for phenotypic expression, results in many progeny mutants of each mutation event. While we can easily define these by use of the TCR gene probes, it would be more economical to develop alternative methods of expression which minimize the multiple mutant copy complication. To this end, we have investigated the possibility of allowing expression with cell aliquots plated in 96 well microtiter dishes by limiting dilution procedures. Such a method of in vitro expression should mean that each mutant colony is the result of an independent mutation event since the

cells were plated prior to cell division. This method has been developed with G_0 phase cultures exposed to 300 rads of gamma irradiation. The cells are incubated for 36-40h with 1 ug/ml PHA to achieve mitogenic activation and then plated at 5 x 10^3 cells/well in growth medium which does not contain PHA. After 3 days incubation, (for phenotypic expression) 50 μl of medium containing TG is added to initiate selection. This time course should result in expression equivalent to that seen on day 5 of mass culture expression. In 4 determinations, the mutant frequencies (x 10^{-6}) for in situ versus mass culture expression were 31.5 versus 49.7, 50.3 versus 32.9, 50.2 versus 46.5, and 28.1 versus 52.6, respectively. The mutant colonies were isolated after this in situ expression and most proved to be independent mutations by TCR gene analysis (Table 6).

TABLE 6 - Molecular nature of hprt mutants induced in vitro by gamma irradiation (300 rads) and grown in LAK supernatant

Expt. # (Mutant Freq.)	Mutants/ Mutations	hprt changes BamHI	HindIII
LS 234 mass culture expression	10 mutants 5 mutations		
($3.2x10^{-6}$sp*, $25.6x10^{-6}$)	4 - exon 1-3 deletion, new fragment	-22,-8,+35kb	-7kb
	3 - exon 2-3 deletion		
	2 - exon 2-3 MW change	-22kb	NC
	1 - exon 2-9 deletion, new fragment	-22,+16kb	NC
		-22,-18,+25kb	ND
LS 259-1 limiting dilution	4 mutants 4 mutations		
	1 - exon 6-9 deletion	-18kb	-17kb
($8.7x10^{-6}$sp, $140.4x10^{-6}$)	1 - NC	NC	NC
	1 - NC	NC	NC
	1 - exon 2-3 deletion	-22kb	NC
LS 259-2 mass culture expression	15 mutants 11 mutations		
	4 - NC	NC	NC
($8.7x10^{-6}$sp, $125.7x10^{-6}$)	2 - exon 2-9 deletion	-22,-18kb	-17kb
	1 - exon 2-3 MW change	-22,+24kb	NC
	1 - exon 6-9 MW change	NC	-17,+15.5kb
	1 - exon 6-9 MW change	NC	-17,+15.5kb
	1 - exon 2-3 MW change	-22,+24kb	NC
	1 - new fragment	+3.5,+3.4kb	NC

	1 - NC	NC	NC
	1 - NC	NC	NC
	1 - NC	NC	NC
	1 - NC	NC	NC
	1 - NC	NC	NC
LS266	7 mutants		
limiting dilution	7 mutations		
	1 - exon 6-9 MW change	-18,+23kb	NC
($8.1x10^{-6}$sp,	1 - total deletion (1?)	-22,-18,-3.7kb	-17,-7,4.9kb
$28.0x10^{-6}$)	1 - exon 6-9 MW change	-18,+14.5kb	-17,+15,+20kb
	1 - NC	NC	NC
	1 - NC	NC	NC
	1 - NC	NC	NC
	1 - NC	NC	NC
		PstI	
LS 323	42 mutants		
limiting dilution	39 mutations		
	3 - exon 3 MW change	-1.2,+4.2,+0.5kb	
($6.9x10^{-6}$sp,	2 - NC	NC	
$50.3x10^{-6}$)	1 - total deletion	-5.1,-4.9,-3.8,-3.0, -2.2,-1.2kb	
	1 - total deletion	-5.1,-4.9,-3.8,-3.0, -2.2,-1.2kb	
	1 - exon 1 deletion	-3.0kb	
	1 - exon 1 MW change	-3.0,+4.5kb	
	1 - exon 1-2 deletion	-3.0,-2.2kb	
	1 - exon 2 MW change	-2.2,+3.7kb	
	1 - exon 2-3 deletion	-2.2,-1.2kb	
	1 - exon 3 MW change	-1.2,+2.3kb	
	1 - exon 3 MW change	-1.2,+9.5kb	
	1 - exon 4 MW change	-5.1,+9.5kb	
	1 - exon 4-6 deletion	-5.1,-4.9kb	
	1 - exon 6 deletion	-4.9kb	
	1 - exon 6 deletion	-4.9kb	
	1 - exon 6 deletion	-4.9kb	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	
	1 - NC	NC	

1 - NC	NC
1 - NC	NC
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1 - NC	NC
1 - NC	NC
1 - NC	NC
1 - NC	NC
1 - NC	NC
1 - NC	NC
1 - NC	NC
1 - NC	NC

*spontaneous frequency

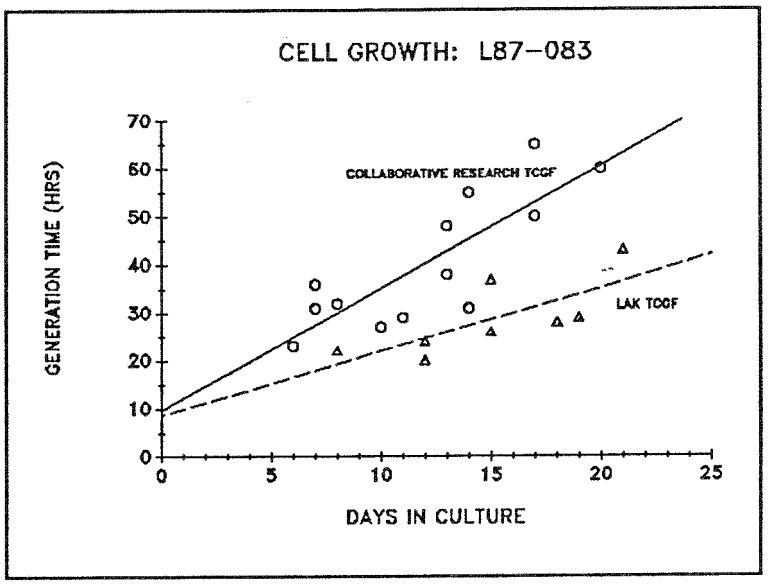
This improvement will allow more rapid characterization of the induced hprt mutations and can be applied to any mutagenic agent at an amount which yields 10-25% relative survival. The need for cell division during the in situ expression does place an upper limit on the number of viable cells which can be plated for 3 days expression to approximately 0.5-1 x 10^4 viable cells/well. The main advantage of this procedure is the more efficient isolation and characterization of independent mutations because sibling colonies are not present. Mutants isolated after mass culture expression (those induced in experiments with mutagenic agents at concentrations giving less than 10% or more than 25% relative survival) will be characterized by TCR gene patterns to determine independence of the mutations.

3. Studies of DNA repair in lymphocyte culture

a. Cell Growth -- There are a number of aspects involved in the mass culture of human T-lymphocytes for in vitro mutation analysis (see above) and for DNA repair studies, many of which we have previously reported (Munzer et al, 1988 and the attached manuscript by Hartshorn, J.N. and Robison, S.H. DNA repair in cultured human T-lymphocytes. Mutat. Res (in press, 1989)) and will be summarized here. In our protocol, T-lymphocytes are isolated from 40 ml of whole blood by Ficoll-Hypaque gradient sedimentation; a typical preparation yields 2-4 x 10^7 lymphocytes. The isolated lymphocytes are washed, assessed for viability by eosin dye exclusion and "primed" with 1 μg/ml of the mitogen PHA for 48 hrs. Following this priming step the activated lymphocytes require two additional factors to sustain growth; T-cell growth factor (TCGF) and feeder cells (x-irradiated TK6 lymphoblasts). Using this system, T-lymphocytes are subcultured at 2-4 day intervals and can be maintained for approximately 40 days as logarithmically growing cells. This basic protocol is used for further analysis of DNA repair.

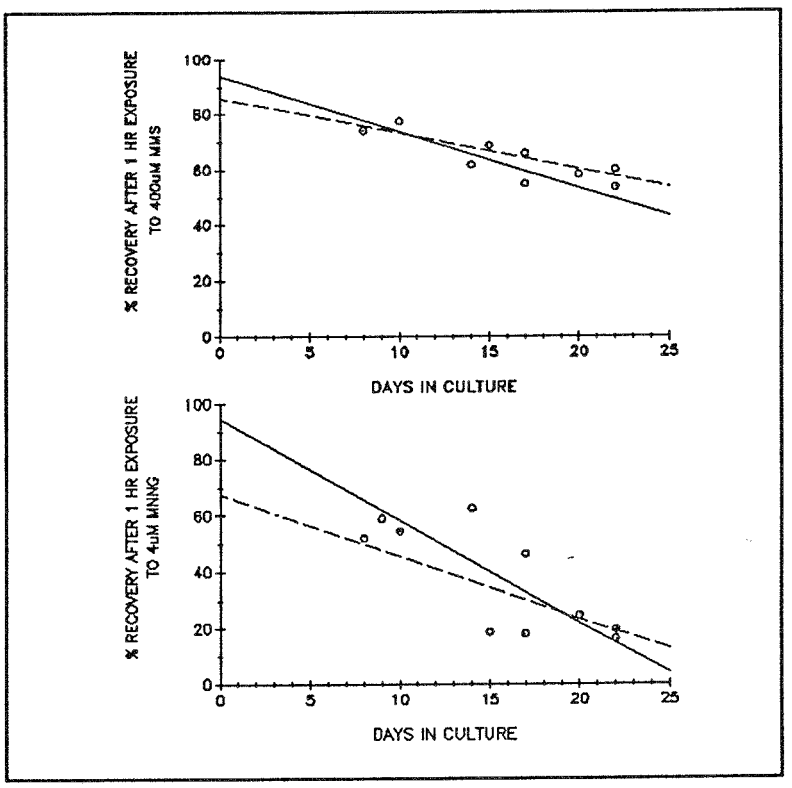
The in vitro culture of T-lymphocytes is dependent upon several factors including seeding density of the cells, concentration of T-cell growth factor in the growth medium, feeder TK-6 cells (8000 rads) and the subculture interval. Each of these factors has been previously considered as reported in Munzer et al, 1988. When in vitro cultures of lymphocytes are maintained for long periods, the generation time of the cells increases from about 20 hrs to about 65 hrs after 25 days in culture as shown in Figure 5.

FIGURE 5 -



In addition, the DNA repair capacity of T-lymphocytes grown <u>in</u> <u>vitro</u> decreases with time in culture, (Figure 6) and there is a differential effect between two different alkylating agents.

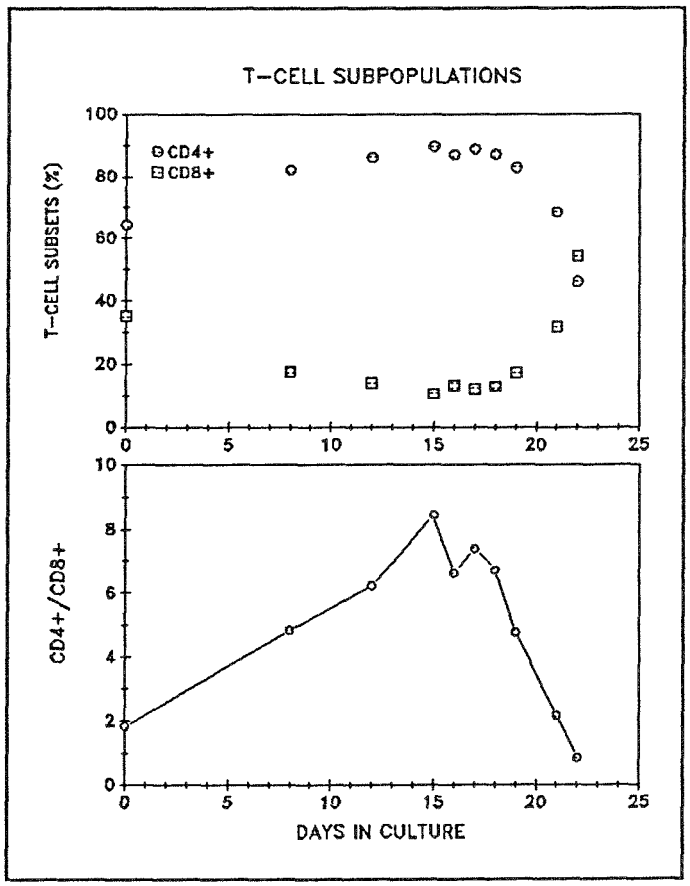
FIGURE 6 -



The repair of DNA lesions induced by the SN_2 agent, MMS, is maintained at a higher level than the repair of the SN_1 agent MNNG. These findings are significant in regard to in vitro mutation assays since repair of mutagenic lesions induced in vitro is affected by time in culture, thus, affecting the potential number of mutants obtained.

Cultured T-lymphocytes are not a homogeneous population, since these cultures contain a mixed group of T-cells containing different surface markers (CD4+/CD8+) and, at early stages, a certain number of B-lymphocytes. We have examined whether the presence of different subsets of T-lymphocytes has any apparent affect on DNA repair capacity. Initially CD4+ T-cells are the primary population until about day 22 in culture when the CD4+/CD8+ ratio reaches about 0.5. Our studies indicate that the two major subsets of lymphocytes $CD4^+$ and $CD8^+$ have no significant differences in DNA repair capacity (Figure 7).

FIGURE 7 -

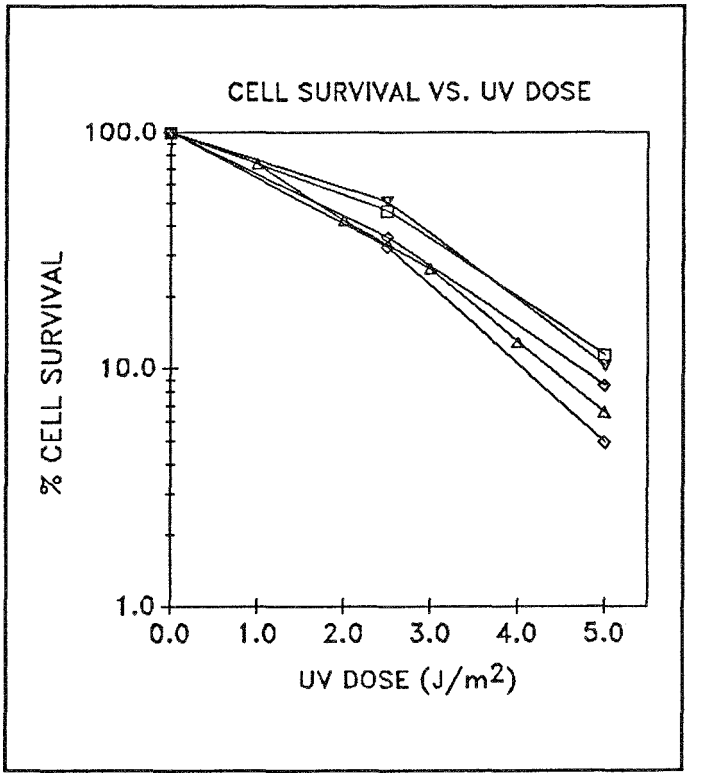


We have also examined generation time, the amount of time in culture and the cell cycle distribution of in vitro cultures of T-lymphocytes and have found that none of these factors adversely affect the ability of T-lymphocytes to repair DNA damage in vitro. The results of these studies can also be found in Hartshorn, J.N. and Robison, S.H. DNA repair in cultured human T-lymphocytes. Mutat. Res (in press, 1989) (appendix).

b. DNA Repair

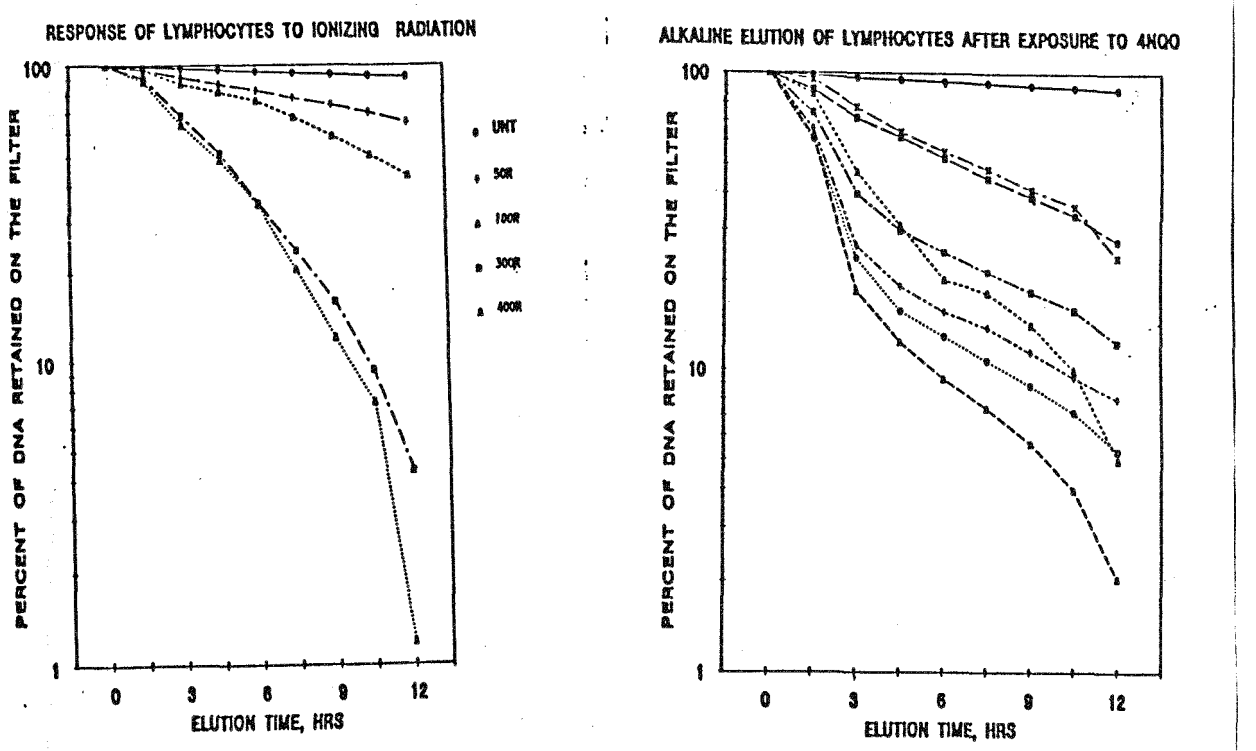
(i) Survival studies -- We have completed cytotoxicity experiments on 4 control subjects. These individuals ranged in age from 36-25 and have no significant disease history or known familial predisposition to disease. The cells are isolated from blood samples, treated with damaging agent or irradiated in PBS-BSA and plated at 2, 10 or 50 cells per well in 96 well plates. Positive colonies are scored 14 days after treatment. In all cases the survival results that we obtained using UV-treated G_o lymphocytes is in agreement with values in the published literature. In all cases there was significant cytotoxicity at UV-irradiation fluences of $2JM^{-2}$ and approximately 90% of the cells were killed at $5JM^{-2}$. The results from these experiments are summarized in figure 8. We are also in the process of defining the concentrations necessary for treatment of cells with alkylating agents.

FIGURE 8 -



(ii) Alkaline elution studies -- We have also developed the methods necessary to study DNA repair after exposure of lymphocytes to DNA damaging agents (Munzer et al, 1988; Hartshorn, J.N. and Robison, S.H. DNA repair in cultured human T-lymphocytes. Mutat. Res (in press, 1989) (appendix). The alkaline elution technique has been adapted for the study of bulk-genomic DNA damage. The results of our studies of DNA repair in bulk-genomic DNA indicate that damage from a variety of different agents can be detected, see Figure 9 and the manuscript by Hartshorn and Robison. We have also studied mass cultures and hprt mutant clones from 2 individuals for DNA repair capacity. The mass cultures repaired 74.2% or 81.7% of the MMS-induced damage after 3 hours and four hprt mutant clones (2 from each individual) had the following levels of repair 3 hours after exposure to MMS of 55.8%, 48.4%, 50.4%, 50.5%, respectively.

FIGURE 9 -



(iii) Gene-specific DNA repair -- Studies of DNA repair in an actively transcribing gene have also been undertaken. We are using the dihydrofolate reductase (DHFR) gene as a model active gene to study the repair of various DNA lesions in T-lymphocyte cultures. Abasic sites can result either spontaneously or from a variety of insults to the DNA molecule including ionizing radiation, chemicals and UV-light. Our preliminary data indicate that there are differences among individuals in the rate of repair of these lesions.

We have completed experiments in our laboratory to determine the initial conditions necessary to detect DNA damage (conversion of N-methylpurines to abasic sites) and its repair in the human DHFR gene. Previous studies have been restricted to Chinese hamster ovary (CHO) cells treated with dimethyl sulfate (DMS) (Drs. P. Hanawalt and D. Scicchitano personal communication and Scicchitano and Hanawalt, 1989). Hence, it was necessary to establish the concentration of MMS required to induce about 1 abasic lesion per 14 kb of DNA in human T-cells. In all experiments the samples are divided into two aliquots; one is treated with methoxyamine for protection of the abasic sites ("plus"), and the other left untreated ("minus") . The DNA samples are heated at 60°C for 6 hrs to liberate N-methylpurines from the DNA, resulting in abasic sites in non-methoxyamine protected ("minus") samples. The DNA samples are incubated at 37°C with alkali to convert any abasic sites to single strand breaks. The DNA samples are then analyzed by alkaline gel electrophoresis, transferred to a nylon membrane, probed with [^{32}P] DHFR cDNA and autoradiographed. The autoradiographs are analyzed by scanning laser densitometry. If abasic sites are present in DNA there will be a significant difference in the intensity between the "plus" (more intense) and "minus" (less intense) bands on autoradiographs probed with the DHFR cDNA. If there are no abasic sites present then the bands should be of equal intensity, provided equal amounts of DNA were loaded into each lane. The amount of DNA in each sample is quantitated by a sensitive fluorometric assay using Hoechst dye #33285, and great care is taken to ensure that exactly the same amount of DNA is loaded in each lane. A ratio of the intensities of the minus/plus bands is calculated and the negative natural logarithm of this ratio is determined. A Poisson distribution is then used to convert this value to the number of abasic sites in 14kb of DNA.

Figures 10A + 10B show autoradiographs of DNA from 4 different individuals T-lymphocytes. A concentration of 1.5mM MMS was required to induce approximately 1 abasic site/14kb of DNA. Lymphocytes from two of the individuals repair most of the lesions, (Figure 10A) and two individuals had considerably lower levels of repair (Figure 10B).

Figure 10A

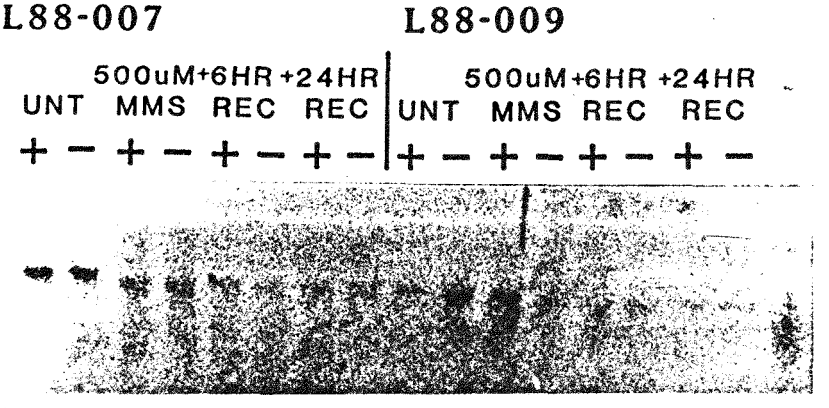
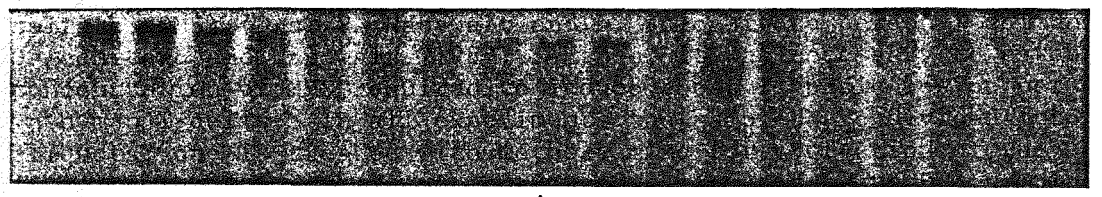


Figure 10B



+	-	+	-	+	-	+	-	+	+	-	+	-	+	-	+	-
Unt		1.5mM MMS		+6Hr Recov		+24Hr Recov		Unt			1.5mM MMS		+6Hr Recov		+24Hr Recov	

L88-012 L88-006A

An interesting finding is that in 3 individuals there was a slight increase in the number of abasic sites after 24hrs (Table 7). This increase is similar to the findings published by Scicchitano and Hanawalt (1989).

TABLE 7 -

CELL LINE	POST-TREATMENT TIME	ABASIC SITES/14KB	PERCENT RECOVERY
L88-009	0HR	1.14	0%
	6HR	0.14	88%
	24HR	0.27	76%
L88-012	0HR	0.94	0%
	6HR	0.009	97%
	24HR	0.00	100%
L88-002	0HR	1.10	0%
	6HR	0.44	60%
	24HR	0.02	98%
L87-013	0HR	0.72	0%
	6HR	0.07	90%
	24HR	0.04	94%
L88-006A	0HR	1.50	0%
	6HR	0.54	64%
	24HR	0.78	48%
L88-007	0HR	0.92	0%
	6HR	0.48	53%
	24HR	0.79	14%
L87-110	0HR	1.24	0%
	6HR	0.47	62%
	24HR	0.43	66%

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