Use of the Asparagine Auxotroph of the L5178Y Murine Leukemia for the Detection of Chemical Mutagens in Vitro and in the Host-Mediated Assay

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Introduction

Identification of the human genetic health hazard from exposure to chemicals of a diverse nature is of significant importance from a public health standpoint. A wide variety of organisms ranging from phage to human cells in culture may be used as tester strains to identify the mutagenic capability of a chemical. While these systems do supply genetically useful information, their major limitation is their inability to consider host biotransformation and other pharmacokinetic parameters. This is of especial importance if the derived data is to have human relevance. While the most ideal situation would be to study man himself, the major limitation to this approach would be of a practical and ethical nature.

Short of studying man, then, *in vivo* mammalian indicator cells would have some obvious advantages. The L5178Y murine leukemia has been relatively unexplored in the area of mutagenicity testing but it has a number of favorable attributes which enhance its utility as an indicator organism. These are as follows: (1) It grows well *in vitro* as a single cell suspension with a doubling time in the range of 10–12 hr (*1*). (2) By a relatively simple technique it can be cloned in soft agar with a plating efficiency of approximately 60–90% with the appearance of macroscopic colonies within 10–14 days (*2*). (3) When implanted into the peritoneal cavity of certain inbred strains of mice, it grows in a reproducible fashion as a single cell ascites suspension (*1*). (4) It has been shown to require an exogenous supply of the amino acid asparagine (asn) (*3*). Thus by cloning the cells in asn-deficient medium, spontaneous or induced mutations to asn-independence can be scored. (5) It possesses a uniform and stable chromosomal complement with a modal number of 42 chromosomes (Fig. 1). (6) It can be harvested from metastatic foci, enabling the study of various organs at risk.

The present communication will summarize our methods for *in vitro* and *in vivo* propagation, the spontaneous mutation rate to asn-independence, the properties of the

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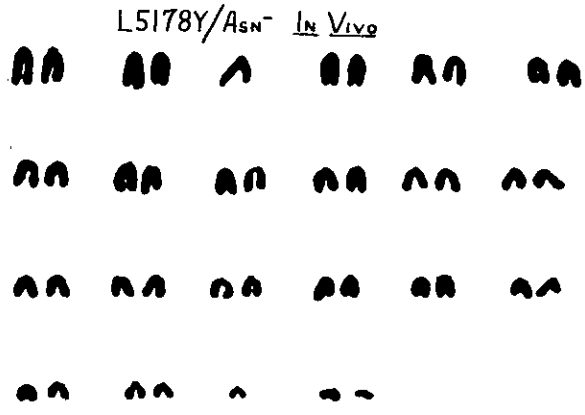

FIGURE 1. Karyotype of L5178Y/asn⁻ ascites cells.

newly derived mutants and the results of mutation induction with known chemical mutagens. The data represent a compilation of experiments independently performed in two separate laboratories (RLC and WPS) by using slightly different techniques and serve to illustrate the reproducibility of studies with this genetic marker. Much of the present material has been published previously (*4–8*).

Methods

Cell Propagation in Vitro

The L5178Y/asn⁻ cell line which was used in both laboratories was obtained in 1969 from Dr. G. A. Fischer. The cells were grown in Fischer's medium (*1*) containing 10 mg/l. of L-asn (designated F^+ medium) and supplemented with 10% horse serum (designated S_{10}) when grown in suspension culture or with 15% serum (S_{15}) when cloned in soft agar. Stock cultures were maintained in continuous exponential growth (doubling time 10–11 hr) by serial dilution two or three times a week. These cultures were maintained in 16 × 125 mm screw-cap tubes in a volume of 5 ml. The cell titer was kept below 4×10^5/ml in order to prevent any loss of viability (*9*) or possible enrichment with mutants as the cells enter the stationary phase of growth.

Asn-less Fischer's medium (F^- medium) was used for the selective growth and cloning of asn-independent cells (L5178Y/asn⁺). The asn concentration of undialyzed horse serum was previously determined (*10*) to be approximately 1–2 mg/l. Thus, the final asn concentration in F^-S_{10} was 0.1–0.2 mg/l. This level is 1–2% of the concentration of asn required for optimal growth of the L5178Y/*asn*⁻ cells (*3*). In F^-S_{10} medium, the cells stop dividing almost immediately and begin to lyse within 24 hr. However, if dialyzed serum is used, lysis begins within 6 hr (*7*).

Larger volumes of cells can be grown under stationary or roller-cell conditions. If stationary conditions are used, optimal growth can occur in 29-oz rectangular prescription bottles kept in the horizontal position. Alternatively, if circular bottles are used, continuous agitated suspension at 6 rpm has been satisfactory with the use of New Brunswick Roller-cell equipment, RC–42 (New Brunswick Scientific Company, New Brunswick, N.J.).

Horse serum and media were purchased from the Grand Island Biological Company, Grand Island, N. Y., and from Microbiological Associates, Bethesda, Md. Prior to purchase, new lots of serum were tested for their ability to support optimal cell growth in suspension culture in addition to providing optimal cloning efficiencies.

Cell Propagation in Vivo

Both the parental L5178Y/asn⁻ cells and mutant L5178Y/asn⁺ substrains grow well as single-cell ascites suspensions in certain inbred strains of mice, notably the BDF_1 and AKD_2F_1 and the strain of origin, DBA_2. For the *in vivo* experiments, male BDF_1 mice were purchased from the Jackson Laboratory, Bar Harbor, Maine, when they weighed 18–20 g and were used for experiments when they weighed approximately 25 g. Stock lines of the L5178Y cells *in vivo* were initiated by intraperitoneal injection of 1×10^6 cells from *in vitro* cultures and maintained by transplantation of 10^6 cells (0.1 ml of a suspension containing 10^7 cells/ml) to new host mice at weekly intervals (*1*).

On occasion, a preexisting mutant may be included in the stock transfer which may lead to enrichment of the stock line with L5178Y/asn$^+$ cells. This problem can be eliminated by transferring 10^4 cells in the stock lines at weekly intervals. It is important to monitor the mouse lines for mutant frequency at periodic intervals to ensure a low background mutant frequency in the mutagenesis experiments.

Cloning Procedures

The soft agar cloning technique as described by Chu and Fischer (*2*) was used with some modifications for the determination of cell viability and frequency of asn-independent mutant cells. Fischer's medium with 15% horse serum was warmed to 37°C and added to the melted Noble agar (50 ml into 5 ml water with 0.11 g agar) which had been cooled to approximately 44°C (the temperature at which the concentrated agar solidifies). The final agar-medium mixture (0.2%) can be kept at 37°C until used, eliminating the need for use of a 44°C bath as described in the original procedure. Prolonged incubation of this medium in loosely capped containers or with small volumes in large containers should be avoided, since the pH increases as CO_2 escapes.

For cloning in 16 × 125 mm culture tubes, 3 ml of the 0.2% agar-medium mixture (at 37°C) and 2 ml of cell suspension (cells diluted in FS_{15}) were added to the tubes at room temperature; the tubes were capped, inverted several times to mix the cells in the medium, then placed upright in an ice-bath for 3–5 min to gel the agar, thereby entrapping the cells in suspension. The cultures were then incubated in the upright position at 37°C. Alternatively, 6 ml of the agar-medium mixture and 4 ml of cell suspension were added to 30-ml Falcon plastic flasks, mixed gently, and the flasks placed in crushed ice for 5 min. The flasks were then incubated in the upright position.

Good cloning results are dependent on careful control of the pH during development of the clones. The medium should be at approximately pH 7.0 at the time of cloning. This may be achieved by adjustment of the pH of the medium when prepared from powder or concentrate (with storage in tightly capped containers to prevent loss of CO_2), or by addition of sterile 0.1*N* HCl to the medium at the time of cloning to bring the medium to a yellow-orange color. Clones develop with poor efficiency when CO_2 is lost and cultures become alkaline. Thus, the cultures must be tightly capped or incubated in a 5% CO_2 incubator. With careful technique, 70–90% cloning efficiency was routinely obtained.

Determination of the asn-independent mutant frequency was performed in a similar manner, except that F^-S_{15} medium was used, and a total of 2.5–5.0 × 10^5 cells were cloned in the 5-ml cultures, or 5 × 10^6 cells in 15 ml in the 30-ml Falcon flasks. Reconstruction experiments indicated that the plating efficiency and recovery of mutants were not affected until the concentration of cells exceeded 5 × 10^5 per 5 ml culture, or 7 × 10^6 per 15 ml culture in the Falcon flasks.

For the *in vivo* studies, ascites cells were removed aseptically by injecting 5 ml of F^-S_{10} through the exposed peritoneum and aspirating as much fluid as possible. The suspension was diluted to 50 ml, centrifuged at room temperature for 10 min at 250*g*, and the cells were washed twice with F^-S_{15}. Finally, the cell pellet was diluted with sufficient F^-S_{15} to provide a cell titer of 5 × 10^6/6 ml.

L5178Y/asn$^+$ colonies which appeared under these conditions grew as rapidly as clones of the L5178Y/asn$^-$ line when cloned in F^+S_{15}. Under optimal conditions, the colonies become visible without magnification within 4–5 days and preliminary counts can be made at about 7 days. Final counts are determined at later times (10–14 days) when the number of clones does not increase further. The mutant frequency was expressed as the number of mutants per 10^6 viable cells, viability being determined by cloning the cells in F^+S_{15}.

When asn-less medium and undialyzed serum are used for the determination of

mutant frequency, some microcolonies also develop. However, colonies composed of L5178Y/asn$^+$ cells are easily distinguished by virtue of their rapid growth, forming large spherical colonies. Cells isolated from the microcolonies and grown in F^+S_{10} did not grow in F^-S_{10}. Furthermore, these did not appear when dialyzed serum was used for the cloning. These small colonies are apparently asn-dependent cells able to grow for a few generations in suboptimal asn concentrations. Nevertheless, the use of dialyzed serum is not advisable because of the variable and lower cloning efficiencies that are obtained.

Growth of the L5178Y/asn$^+$ Substrains

L5178Y/asn$^+$ cells derived from clones in F^-S_{15} are immediately able to grow in asn-free medium with approximately the same doubling time as the parental, L5178Y/asn$^-$ line. Thus, clones which arise under these conditions can be tested for asn independence by picking the clone out of the culture with a sterile Pasteur pipet and suspending the cells in 5 ml of F^-S_{10}. Under these conditions, L5178Y/asn$^-$ cells fail to grow and soon begin to lyse, while cells from an L5178Y/asn$^+$ clone grow about as rapidly as when a comparable number of cells from culture are diluted to this concentration. For example, clones picked at 7 days, containing approximately 5×10^4 cells, typically yielded 2 to 3×10^6 cells after three days growth, indicating an average doubling time of no more than 12 hr. This method can be used as a rapid screen to verify that the clones observed are asn$^+$ mutants, since rapid growth (asn$^+$ cells) versus cell lysis (asn$^-$ cells) is easily determined by simple observation of the cultures.

Asparagine Synthetase Assays

Two methods of measurement of the capacity of various L5178Y/asn$^+$ mutant cell lines for asn synthesis were used in previous reports (*6,7*). The methods for measurement of the level of asn synthetase activity in subcellular preparations and of the accumulation of asn during short-term incubation of whole cells at high density were previously described by Chou and Handschumacher (*11*).

Results

The spontaneous mutation rate to asn independence was determined by using the fluctuation test as described by Luria and Delbrück (*12*). By using eq. (*8*) of Luria and Delbrück (*11*) with the aid of tables as described by Capizzi and Jameson (*13*), the *in vitro* mutation rate of L5178Y/asn$^-$ to L5178Y/asn$^+$ was found to be 7.4 and 9×10^{-7}/cell/generation in two independent investigations (RLC and WPS, respectively). Fluctuation tests performed with clones adapted to *in vivo* growth yielded a mutation rate of 9.9×10^{-7}/cell/ generation.

Mutations to asn independence have been induced by mutagens known to produce either base pair substitution or frameshift mutations. Dose-response data for the bifunctional alkylating agent sulfur mustard (SM) are shown in Table 1. L5178Y/asn$^-$ cells were plated in F^-S_{15} immediately after 30 min of exposure to sulfur mustard. A nonlethal dose of SM (0.001 μg/ml) failed to increase the mutant frequency. However, as cytotoxicity increased, so did the mutant frequency.

The induction of mutation with other known mutagens is shown in Table 2. L5178Y/asn$^-$ were exposed to various agents *in vitro*, then washed and incubated in fresh,

Table 1. Sulfur mustard-induced mutants to asparagine independence assayed on day of treatment.[a]

Dose, μg/ml	MF $\times 10^{-6}$ survivors	
	Control	Treated
0.001	6.4	8.3
	1.5	3.0
0.02	11.3	15.9
0.06	1.6	23.7
0.12	1.2	155.8
	1.6	143.4

[a] L5178Y/asn$^-$ were exposed to sulfur mustard for 30 min, washed twice, then cloned for the determination of viability and mutant frequency (MF).

Table 2. Induced mutants to asn independence by *in vitro* treatment of L5178Y/asn⁻[a].

Agent	Dose, *M*	Exposure time, hr	Viability %	Expression time, hr	Mutants per 10^6 viable cells[b]
Control					
MNNG	5×10^{-7}	2	17	45	2.6 (5)
Control					2.1 (2)
EMS	6×10^{-3}	2	38	45	23 (2)
Control					0.9 (2)
EMS	10^{-2}	2	24	42	13 (2)
Control					1.5 (5)
ICR–372	2×10^{-6}	20	18	48	24.9 (5)

[a] After exposure of the L5178Y/asn⁻ to the designated agents the cells were washed twice and resuspended in F^+S_{10}. Following an "expression" time the cells were washed then cloned for viability and mutant frequency.

[b] (———) Numbers in parentheses, indicate the number of replicate determinations.

complete medium for 42–48 hr to allow "expression" of the mutation prior to cloning in the selection medium (F^-S_{15}). An increase in mutant frequency was produced by the alkylating agents ethyl methanesulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as well as the frameshift mutagen ICR–372, 2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]1-azaacridine dihydrochloride.

The utility of the host-mediated assay with the use of the L5178Y ascites tumor is indicated with studies with dimethyl nitrosamine (DMNA). Four days after the intraperitoneal implantation of the L5178Y/asn⁻, the mice were treated with a single subcutaneous dose of DMNA. At 6 and 96 hr after treatment, the cells were harvested, washed twice with F^-S_{15}, and cloned for viability in F^+S_{15} and for mutant frequency in F^-S_{15}. As is shown in Figure 2, when the cells were assayed 6 hr after treatment, no increase in mutants was produced by 2.5 mg/kg, and only a slight increase was produced by 5 and 10 mg/kg. However, when the cells were assayed 4 days after treatment, doses of 2.5, 5, and 10 mg/kg increased the mutant frequency by 33, 160, and 900, respectively. Comparable exposure of the L5178Y/asn⁻ to DMNA *in vitro* failed to produce any cytotoxic or mutagenic effects.

Other known mutagens, EMS, MMS, and MNNG, were also effective in increasing the asn⁺ frequency when administered in the host mediated assay.

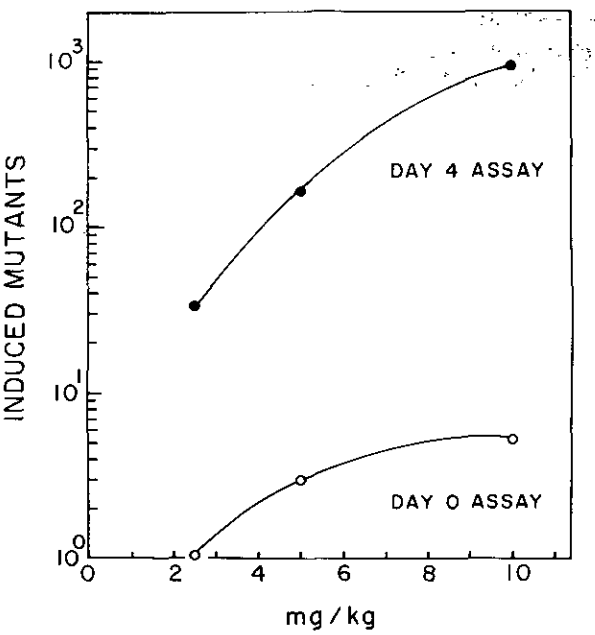

FIGURE 2. DMNA-induced mutants to asn-independence. Mice were treated with a single subcutaneous dose of DMNA 4 days after the intraperitoneal implantation of 10^6 L5178Y/asn⁻ cells. Cells were harvested from the mice and cloned for viability and mutant frequency at 6 hr (day 0 assay) and 4 days (day 4 assay) after treatment.

In order to be sure that neither the auxotroph nor the prototroph was at an advantage or disadvantage to each other because of

selective cytotoxicity, clonal isolates of both sublines were tested with equivalent doses of each chemical and no significant difference was observed.

Spontaneous asn$^+$ mutants have been studied in detail with respect to growth kinetics and asparagine synthetase levels (*7*). Although asn$^+$ clones extracted from F^-S_{15} grow at near the optimal rate in F^-S_{10} upon serial passage, it was repeatedly observed that the growth rate was not identical to the L5178Y/asn$^-$ for the first few weeks passage in the asn-less medium. To further characterize the properties of newly derived spontaneous mutants, clonal isolates were maintained under nonselective conditions (i.e., in F^+S_{10}) except during the selective cloning step. This procedure revealed that the initial mutation does not impart total asn independence. This is evident by a slower, nonpopulation-dependent growth rate (20–24 hr) of the cells when subcultured in asn-less medium. They resume the 10–11 hr doubling time within 16–24 hr. This was observed in the newly isolated clones and has remained unchanged after many passages in F^+S_{10}. The serial passage of mutant substrains in F^-S_{10} produced asn$^+$ lines whose growth was identical in the presence and absence of asn.

The specific activity of the enzyme, asparagine synthetase, correlated with these cell growth patterns. The enzyme activity in newly isolated clones and in the progeny of mutant clones maintained in F^+S_{10} for periods in excess of a year was approximately 4 nmole/mg protein/hr compared to <0.7 nmole/mg protein$^-$ hr in the parental, L5178Y/asn$^-$ cells. However, the increase in growth rate after continuous subculture in F^-S_{10} was associated with a significant increase in the asn synthetase activity (15–30 nmole asn/mg protein-hr in various fully adapted asn$^+$ lines). This level was not decreased by growing the cells in asn-supplemented medium (F^+S_{10}).

Thus the original mutation is to an intermediate level of asn synthetase and the further transition to complete asn-independence may require further mutation and selection. Growth studies and asn synthetase assays on mutants induced by MNNG, EMS, and ICR–372 indicated that these mutants had the same characteristics as the initial spontaneous mutants.

Discussion

The applicability and utility of various nonmammalian and mammalian tester strains for the detection of chemical mutagens have been discussed in several recent texts (*14–16*). Although the L5178Y murine leukemia has not been extensively investigated, several recent reports have indicated the applicability of the thymidine kinase locus (*17*) and resistance to MTX, Ara-c and IUDR (*18*) as genetic markers for the detection of chemical mutagens.

The susceptibility of the asn locus to mutation by chemicals known to produce frameshift mutations and mutations by base-pair substitutions suggests that the observed change in phenotype from auxotrophy to prototrophy might not be a "reverse" mutation but rather a "forward" mutation involving the inactivation of a repressor or inhibitor. This observed mutability of the asn locus by known chemical mutagens warrants further exploration.

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