The Host-Mediated Mammalian Cell Assay

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Abstract

The mutation of L5178Y$_c$ and L5178Y$_d$ cells in culture provides the capability of testing known compounds for mutagenic activity. Since the cells also reproduce in the ascitic cavity of genetically compatible mice and are single cell infective, a murine host-mediated assay may also be done. The procedures required for the mutagenicity test in culture and in mice are outlined in (A) and (B). The soft-agar cloning technique is described in (C) and recent developments in culture techniques are presented in (D) of this article.

(A) Mutagenicity tests in culture

(1) *Toxicity test*

(a) *General procedure:* Treatment of cells with the chemical: L5178Y$_c$ or L5178Y$_d$ cells [1] are maintained in culture in the exponential phase of growth having the medium changed twice weekly. Cells should be stored at $-70\,^{\circ}C$ as described later. The doubling time should be from 10.5 to 13.5 hours and the number of cells per ml should not exceed 4×10^5. Cells from culture should be at a density of 2 to 4×10^5 cells/ml when used, and are diluted and maintained at 37 °C except where noted. The agent to be tested is dissolved in distilled water at a concentration of 100 mg/ml when possible. If the agent does not go into the solution, other approaches may be tried (ex. NaOH, HCl, dimethyl sulfoxide). In many instances it may be essential to add the drug as a suspension (ex. sucrose). Drug is added to 5 ml of L5178Y$_c$ or L5178Y$_d$ cells (5×10^4 cells/ml) in a dilution series (ex. 30, 10, 3, 1, 0.3 mg per tube) during the exponential phase of growth in 37 °C Fischer's growth medium containing 10% horse serum [(FMS) (Grand Island Biological Company, Grand Island, New York)]. Cells are incubated with drug for 4 hours and 18 hours. The test tubes (16×125 mm) may be plastic or glass screw-cap tubes. The drug is removed by centrifugation at about $800 \times g$ in a Model CL International Centrifuge for 3 minutes. The cells are resuspended in 5 ml of drug-free FMS and the washing procedure is repeated once. In order to determine cell number, the small cell aggregates are dispersed by pipetting and expelling the cells against the wall of the culture tube three times using a 5-ml pipette. 2 ml of the single cell suspension are diluted with 8 ml of 0.9% NaCl and the cells counted in a Coulter Particle Counter, Model A or Model B.

(b) *Determination of cell viability:* Cell viability is determined by soft-agar cloning method (see C). Eighty cells of untreated control and drug-treated cells are added to each of four 16×125 mm screw-cap tubes to which 3.0 ml of agar solution has been distributed. Control cells must undergo approximately 1.3 doublings in 18 hours. The cells are incubated upright at 37° for 10 days to permit optimum colony formation. At the end of the incubation the colonies are counted with a Quebec Colony Counter. The average number of colonies found in each control tube should range from 48 to 68 colonies (the cloning efficiency should range from 60 to 85%).

(c) *Calculation of results:* All colony counts are normalized to 100% cloning efficiency of the controls. The cloning efficiency is plotted on a linear scale and the level of drug on a logarithmic scale. The points are fitted by eye to a straight line, then the concentration of drug which would have killed 80% of the L5178Y$_c$ or L5178Y$_d$ cells is determined. This dose (80% kill) is the drug level to be used in the subsequent mutagenesis experiment. Should cell kill be more than 80% at the lowest drug level tested, the study will be

repeated using lower drug levels. Should no cell kill occur at the highest drug level, the highest level will be used in the mutagenicity test.

(2) *Mutagenicity test in culture*

(a) *General procedure:* In the mutagenicity test, L5178Y$_c$ or L5178Y$_d$ cells will be exposed to the chemical for 4 hours and for 18 hours at a drug level which produces approximately 80% cell kill. After removal of the drug the cells will be incubated in growth medium at 37° for 96 hours allowing expression of the mutant. Mutant frequency will then be determined for markers which confer resistance either to cytosine arabinoside, to methotrexate or to thymidine. A significant increase in mutant frequency for any of these three markers will suggest that the chemical thus studied is mutagenic. A total of six loci are studied in this system with mutation rates ranging from 2×10^{-5} to 1×10^{-7}. An increased mutant frequency in treated cells must be repeated in order to detect the occasional fluctuations of mutant frequencies which might occur in different cell populations, thus resulting in false positives. Reconstruction experiments are done in order to exclude the possibility that the agent selectively kills mutant or wild type cells and the cloning efficiency in culture of selected mutant cells has been determined and is similar to the cloning efficiency of sensitive cells [2]. Stocks are replaced from the bank [3] on successive 120 day intervals.

(b) *Exposure to chemical:* 5.0×10^6 L5178Y$_c$ or L5178Y$_d$ cells in 5 ml of FMS are exposed, in duplicate to the drug concentration which produces approximately 80% kill. There are two time periods of exposure to agent; 4 hours and 18 hours. At the end of the incubation period the cells, including controls, are centrifuged at $800 \times g$ in a Model CL International Centrifuge for 3 minutes, and the supernatant medium is decanted. The cells are resuspended in 5 ml of growth medium. This procedure is repeated once.

(c) *Expression of mutants:* The washed cells are dispersed by drawing them into a 5 ml pipette and expelling the contents against the side of the tube. This process is repeated three times. The cells are counted by adding 2 ml of the cells to 8 ml of 0.9% NaCl and counted. The cells are then diluted in 100 ml screw-cap serum bottles and incubated at 37° in a final volume of 35.0 ml. Control and treated cultures are diluted to 1×10^5 cells per ml daily for three days when needed.

(d) *Recovery of mutants:* Mutants are recovered after incubation for 72 hours if the control drug-free cultures have shown a doubling time from 10.5 to 13.5 hours. After counting the cells a solution of 10 ml containing 2.0×10^5 cells per ml is prepared from each incubation. Antimetabolites are added to the concentrated agar kept at 44°C in a water bath as in the host-mediated assay. Four tubes are set up for each antimetabolite. 2 ml of cells, 2×10^5 cells/ml, are added to each tube containing 3.0 ml of agar solution. After gentle mixing the cells are immersed in an ice bath for 3–5 minutes. The tubes are then incubated upright for 14 days at 37°, since mutant colony growth is often slightly inhibited by the antimetabolite. Colonies which appear in the presence of the antimetabolites will be mutants. In order to determine cloning efficiency, 80 cells per tube (4 tubes) from untreated control cells are placed in soft agar and incubated for 10 days. The number of mutants are normalized by multiplying the factor required to bring the cloning efficiency of the controls to 100% (normalization of mutant frequency to 100% recovery). Mutant frequency (m.f.) is determined by the following formula:

$$\text{m.f.} = \frac{\text{Normalized number of mutants}}{\text{Total number of cells added}}$$

(B) L5178Y$_c$ and L5178Y$_d$ mutagenesis test in host-mediated assay system

(1) *General remarks*

The properties of murine leukemic lymphoblasts, L5178Y$_c$ and L5178Y$_d$, which grow both in the animal and in culture provide an animal-mediated assay system for the detection of chemical mutagens using a mammalian target cell. Using dilution techniques [3], the single cell infectivity of appropriate mutants has been done and resembles the cloning efficiency of the cell line of origin [2]. In addition, reconstruction experiments are essential for 'active' compounds in order to determine whether the agent has a selective killing effect on mutant or wild type cells.

The procedure of the mutagenesis test is outlined as follows:

(2) *Toxicity test*

Each mouse (DBA/2J or AKR × DBA$_2$, Cumberland Farms) is injected intraperitoneally with 10^6 L5178Y$_c$ or L5178Y$_d$ cells in approx-

imately 0.5 ml growth medium (FMS). Stocks of cells for injection are maintained in mice by intraperitoneal injection of 10^3 L5178Y$_c$ or L5178Y$_d$ cells into each of 5 mice. Transplants are made every 11 to 14 days. Cells can be stored at −70 °C [3]. 10% dimethyl sulfoxide rather than glycerol in FMS is used to suspend the cells during freezing and storage. Stocks are replaced from the bank on successive 120 day intervals. Groups of mice are treated in each concentration of test compound. 3 days after the inoculation, each group receives the drug preferably dissolved in 0.1 ml 0.9% NaCl. Each of the control group of 4 mice receives 0.1 ml 0.9% NaCl. When necessary, other solvents for drugs could be used along with appropriate controls. Various routes of administration of drug are possible. The subcutaneous injection of 0.1 ml of drug on the back of the animal is one convenient route. The drug levels used are 1500, 500, 150 and 50 mg per kg. If needed the toxicity study will be repeated using a narrow range of drug. Should toxicity (death or weight loss) be evident 3 days after drug injection at the lowest drug level tested, the toxicity test should be repeated. If no toxicity is observed at the highest level used, the highest dosage will be used in a mutagenicity experiment. A second method of drug treatment is to use a subacute dosage of the host by daily injection of the agent for a period of 3 weeks prior to the injection of 1×10^6 target cells followed by administration of agent on days 3, 4 and 5. In this schedule, cells from the animals are cultured for 18 to 24 hours as described later in this subsection. Due to limited solubility of the drug, it may be essential to inject drug suspensions. To estimate target cell toxicity, cells are quantitatively recovered from the host ascitic fluid on day 6 and the cells counted. A low number of tumor cells suggests that the agent may possess anticancer properties.

(3) *Mutagenicity test*

Groups of two mice are injected with 1×10^6 L5178Y$_c$ or L5178Y$_d$ cells per mouse. 3 days after the inoculation, the drug is given to a test group of four mice for each agent. The dosage of drug, the schedule and the route of administration are decided for the particular experiment after considering host toxicity and target cell toxicity. The control group is treated in the same way without drug.

Three days after the drug, cells from ascitic fluid are recovered asceptically by i.p. injection of 5.0 ml FMS. The cell samples are diluted with 5 ml of growth medium and immediately centrifuged at $800 \times g$ for 3 minutes. The supernatants are decanted, cells are resuspended in 5 ml of FMS and cell concentrations determined. Cells from each host may be immediately diluted in soft agar and cell viability and mutant frequency determined for each animal. Cells from comparable animals are not pooled. Alternatively, one sample from each mouse is diluted to 1×10^5 cells per ml in a volume of 35 ml growth medium in a 100 ml screw-cap bottle. The cells are incubated at 37 °C for 24 hours to deplete contaminating cells from the host. The second method of subacute dosage may also be used.

Mutants are determined by the soft-agar cloning method (see C) as follows: Three antimetabolites are used to detect the three markers, 0.55 ml of 10^{-5} *M* methotrexate is added to 55 ml of the concentrated 0.2% agar growth medium kept in a 44 °C water bath. 1.28 ml of 0.1 *M* thymidine and 0.92 ml of 10^{-4} cytosine arabinoside are added to bottles containing 55 ml agar medium at 44 °C. 3 ml of the agar media are distributed into 16×125 mm culture tubes with screw caps. 2 ml of single cell suspensions (2×10^5 cells per ml) are added to each of four tubes at room temperature. The final concentration of methotrexate is 6×10^{-8} *M*, thymidine 1.4×10^{-3} *M*, and cytosine arabinoside 1.0×10^{-6} *M*. The tubes are mixed gently and quickly, kept in ice for 3–5 minutes and incubated upright at 37 °C. In order to determine the cloning efficiency for all cell samples, 80 untreated control cells per tube are also cloned in the agar medium without antimetabolites. The spontaneous mutation rates in culture or in animals agree well when calculated from mutant frequency at a single point in time and assuming immediate expression of the mutants with no selective advantage during reproduction for wild type or mutants [2].

The colonies in medium containing antimetabolites are counted on the 14th day of incubation. Colonies grown in the agar medium without antimetabolites are counted on the 10th day of incubation. All the colony counts are averaged and normalized to 100% cloning efficiency of the controls, and the mutant frequency of the treated sample is compared to that of control which should be approximately 1×10^{-6}. The mutant frequency in litter-mates will be variable since the 1×10^6 cells injected may contain different numbers of pre-existing mutants.

Therefore, if results suggest mutagenic activity of a chemical, the test must be repeated until the results are clear.

(C) The soft-agar coloning method [4]

(1) *General remarks*

The soft-agar cloning method provides a convenient system for the recovery of mutant progeny in culture, as round tridimensional colonies. The advantages of this system in which cloning efficiency is linear in the range of 120 to 3 colonies per tube are: (1) Semidilute agar (0.2%) can be maintained at room temperature for 15 to 30 minutes without gel formation. This provides a comfortable flexibility of time for the execution of experiments. (2) Up to 80 colonies can be recovered and conveniently counted in a single culture tube. This compares favorably with those cloning systems in which petri dishes or other types of containers are used for the formation of tridimensional colonies. In addition, the label on each tube can be different and errors avoided. (3) Cloning in round culture tubes minimizes the bench space required for the execution of experiments. (4) Contaminants (ex. Mycoplasma) can be detected as an excess number of colonies of very small size which might appear 2 days prior to the appearance of visible L5178Y colonies. Such contaminants can be isolated and identified. Extracellular Mycoplasma is acutely lytic for L5178 cells but not for certain of its sublines (ex. $TK^{+}/-$ of D. CLIVE, W. G. FLAMM and M. R. MACHESKO, Mut. Res. *14*, proof copy 001, 1972). (5) The dilute agar can flow through the aperture of a Coulter Particle Counter and enumerated in some situations. Most agar lots (Difco-Noble) form satisfactory gels at a concentration of 0.07%. (6) Colonies can readily be centrifuged through the agar, collected as a pellet and processed for further study.

(2) *Experimental procedures*

L5178Y leukemic cells in their exponential phase of growth at 37 °C are prepared from culture at a cell density of 2 to 4×10^5 per ml and maintained at 37 °C except where noted. The cell number is determined with a Coulter Particle Counter (Model A or Model B). A group of control cells is diluted to provide 50 cells per ml in a volume of 10.0 ml of FM containing 15% horse serum. 2 ml portions from each cell suspension are added to each of four culture tubes (16×125 mm) to which 3.0 ml of agar solution has been distributed. The final concentration of agar is 0.12%.

Preparation of agar solution: 6.5 ml of water is added to 110 mg agar (Difco-Noble) and autoclaved for 15 minutes in a 100 ml screw-cap flask and can be stored up to 14 days at approximately 4 °C. The sterile solution of agar is remelted in a boiling water bath for 30 minutes, cooled in a 60 °C water bath for 30 minutes prior to the addition of 50.0 ml of Fischer's medium containing 15% horse serum that has been kept in a 44 °C water bath for 5 to 10 minutes. The concentrated agar (2.2%) should be colorless or slightly tinted (browning).

After addition of 2 ml cell suspension, the caps are tightened and the tubes inverted gently and quickly in order to mix the agar with the cell suspension. In order to obtain a uniform gel, the tubes are kept upright in an ice bath for 3 to 5 minutes. The tubes are incubated vertically on foam rubber mats, 3/4 inches thick which insulates the tubes from vibration during the incubation. Tubes are re-tightened 24 hours later. Colony counts will be made on day 10 or on day 14 using an AO Quebec darkfield colony counter. When mutants are to be recovered, the drugs are added to the concentrated agar as described above and 4×10^5 cells are added to each of four tubes. All colonies are quantitatively and conveniently recovered with an empty sterile Pasteur pipette and are readily dispersed in liquid medium to a single cell suspension, using a sterile 5-ml pipette.

(D) Recent developments in culture techniques for murine leukemic cells

Culture techniques for a variety of murine leukemic cell lines have been described [5]. Since the time of that report, significant advances in culture techniques have been made [6].

Horse serum (Grand Island Biological Company, Grand Island, New York) before use was assayed: (1) for growth-promoting activity by outgrowth method [5] and (2) for ability to promote colony-formation [6] by the soft-agar cloning technique [4], using murine leukemic cells (L5178Y). Cells in the exponential phase of growth at 37 °C are prepared from culture at a cell density of 2 to 4×10^5 per ml and maintained at 37 °C except where noted. Each assay is repeated in a separate experiment. Serum of known unit activity is used as a control. For the outgrowth method, 5.0 ml of cells from culture were centrifuged at approximately $800 \times g$ for

5 minutes and resuspended in 5.0 ml of serum free Fischer's medium (FM) and distributed in duplicate into glass culture tubes (16 × 125 mm) containing, respectively, 10, 4, 2, and 1% serum by volume and an inoculum of 5×10^3 cells per ml, 5.0 ml per tube and incubated for a period of 72 hours. The cell number is then determined with a Coulter Counter, Model B. The logarithm of cell number is plotted as a function of serum concentration (linear) and one unit of serum is defined as that level which permits the reproduction of one-half the cell doublings maximally obtained. Acceptable lots of serum have a unit activity less than 3%.

The assay for the cloning activity of serum is accomplished by the method of FISCHER and CHU [6]. The inoculum of L5178Y cells is prepared from cultures as specified above. After determination of the cell number, cells are diluted 1:100 in a final volume of 10.0 ml of FM. Aliquots containing 300 cells are diluted again in 10.0 ml FM containing 15, 10, 7 and 4% of serum by volume. 2 ml of this cell suspension was added to 3.0 ml of FM containing appropriate concentrations of serum and 0.2% agar giving a final agar concentration of 0.12% [4]. Difco-Noble agar (110 mg) in 5.0 ml of deionized glass distilled water (2.2%) is sterilized by autoclave (15 lbs., 250 F, 15 minutes) and stored at 4°C. Before dilution the agar is redissolved by heating in a boiling water bath for 30 minutes, cooled and maintained at 60° for a period of time not exceeding 30 minutes and transferred to a 44°C water bath for approximately 10 minutes in order to minimize the inactivation of heat labile fractions contained in horse serum. 1.3 ml of this agar is diluted to 0.2% by the addition of 13.0 ml FM containing the desired concentrations of horse serum. The pH is adjusted to approximately 6.9 by the addition of sterile 0.1 N HCl. The 0.2% nutrient agar is distributed in 3.0 ml aliquots into four glass screw-cap culture tubes (16 × 125 mm) for each serum concentration. The cells (30/ml) are removed from the incubator, taken to the laboratory, and added to the 0.2% agar in 2.0 ml FM containing appropriate concentrations of serum. The tubes are mixed quickly and gently, and placed in an ice bath for 5 minutes in order to hasten gel formation. The tubes are incubated vertically on foam rubber mats, 3/4 of an inch thick in order to minimize vibration of the gel during the period of incubation. Tubes are transferred to a 37°C incubator and re-tightened 18 to 24 hours later. After incubation at 37°C for 10 days, the colonies are counted with an AO Quebec darkfield colony counter. The cloning efficiency (% recovery) is plotted as a linear function of serum concentration. One unit of serum is defined as that concentration which permits the formation of 50% of the colonies maximally obtained. Acceptable lots of serum have a cloning efficiency of 60–85% at high serum concentrations (15% and perhaps 10%) and a unit activity of 7% or less. Serum is stored by the manufacturer in large quantities (20–40 l) for a period of no longer than 3 months, received in small quantities, stored at −16°C and used within a period of 3 weeks.

Glassware used in cell culture work is prepared according to standard procedures: thorough cleaning in strong acid (concentrated sulfuric acid saturated with potassium dichromate or strong base (approximately 1 N NaOH), rinsed five times in distilled water and finally rinsed five times in deionized and glass re-distilled water. Water for use in the preparation of medium and chemicals is prepared by three successive steps: (1) a steam-heated water still, (2) deionization using a mixed-bed resin and (3) final re-distillation in an all glass water still. FM (10× concentrate, Grand Island Biological Co., Grand Island, New York) is diluted with sterile water to which dry sterile $NaHCO_3$ has been added. Before use, medium is assayed using L5178Y cells. Serum of known unit activity is added to give a final concentration of 10% (FMS) to an inoculum of 2×10^3 cells per ml and a 6 point growth curve obtained within a time range of 72 hours. The best fit, straight line is determined using a programm and an Olivetti Computer. For the control, a prior preparation of FM is used. The best fit of a linear regression of log-transformed cell concentration on incubation time is generated with an Olivetti Programma 101 Computer. It is from this regression line that the cell doubling time is calculated. When different media are being compared, the 95 per cent confidence belts of the different regression lines are used to ascertain that the cell doubling time using the new medium is within 1.5 hours of the doubling time using the control medium. Medium (10×) is received and stored at 4°C and used within a time period of 40 days.

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