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Teratological Research Using *In Vitro* Systems. IV. Cells in Culture

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Several *in vitro* cellular systems designed to screen agents for teratogenic potential are described in this report. These assays were selected from a review of literature published through the spring of 1986 that generated over 100 references on teratological research using cell-based systems. Some of the assays have a broader application than others, but most require confirmation by one or more additional complementary tests because of the specificity of the teratogenic mechanism the assays are investigating. Included are systems that use analysis of tumor cell attachment; intercellular communication; growth of human embryonic palatal mesenchyme cells; progesterone production in porcine granulosa cells; differentiation of embryonic neural crest, limb bud, midbrain, and Drosophila cells; and differentiation of tumor cells. Because of the dynamic nature of cell culture work, the group of assays listed here should not be viewed as encompassing all cell systems of value with regard to teratogenicity testing; instead, the list represents several of the more prominent systems now being evaluated by the scientific community.

Inhibition of Tumor Cell Attachment to Concanavalin A-Coated Surfaces

Introduction

Interactions between embryonic cells have long been thought to be critical factors in embryonic development. The breakdown of these intracellular communications by physical intervention or genetic defects are thought to be associated with developmental anomalies. Braun and associates (*1*) have described an *in vitro* cell-to-surface recognition system using mouse ascites tumor cells to screen for potential teratogens. The use of tumor cells in this type of assay has several advantages: tumor cells are readily available, homogeneous, easy to prepare, and share many characteristics with embryonic cells. One characteristic that transformed fibroblasts, tumor cells, and some embryonic cells share is the ability to attach themselves to lectin-coated plastic surfaces, whereas normal fibroblasts attach poorly. This difference has been exploited as a means of differentiating chemicals that alter cell-surface interactions from those that do not. Tumor cells have been shown to attach rapidly and irreversibly to lectin-coated plastic disks because of interactions between carbohydrate moieties on the cell membrane and lectin receptors on the plastic surface. Requirements include metabolic energy and an intact cell surface. Attachment can be quantitated by the use of radiolabeled cells. Braun and Horowicz (*2*) have proposed that any agent capable of interfering with lectin-mediated attachment can be expected to interfere with morphogenic recognition processes and lead to congenital malformations.

Methodology

This assay entails inoculating mice with ascitic mouse ovarian tumor cells 1 week before the assay is performed. The cells are radioactively labeled by intraperitoneal injection with tritiated thymidine about 12 hr before use. Cells are then harvested and allowed to incubate at 37°C for 30 min in a Petri dish containing the test compound in solution. Since attachment is dependent upon pH, the test solution is titrated to 7.4 before exposure to the cells. Several polyethylene disks coated with concanavalin A are placed in the suspension for 20 min at room temperature, then removed, washed in saline, and counted in Aquasol (New England Nuclear, Boston, MA) with a liquid scintillation counter. A set of eight or nine concentrations is used for each compound tested. Concentrations are maintained below overtly toxic levels, as determined by trypan blue exclusion. Braun has suggested that in the event cytotoxicity is noted, incubation with 5% fetal calf serum (FCS) should be attempted, since a number of chemicals found to be toxic in saline were later shown to be inhibitory in serum. FCS was found to protect the cell from toxicity at concentrations of agent that inhibited attachment. Compounds that are neither toxic nor inhibitory at their solubility limit are reassayed at longer incubation intervals with the test compound (1–2 hr). Each

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data point is obtained in triplicate. Counts of adherent cells on the three disks are averaged and the number of counts adherent to bovine serum albumin-coated disks is subtracted as the control. Results are expressed as a percentage of attachment by untreated cells to concanavalin A-coated disks (*1,3,4*). Another measure used to assess an agent's effect is the concentration required in order to inhibit attachment by 50% (ID_{50}).

Critical Review

Attempts have been made to validate this system with over 100 chemicals in two basic categories, drugs and pesticides. The lowest effective dose reported for a teratogen was used to estimate teratogenic potency; in considering nonteratogens, the highest reported nonteratogenic dose was used. Inhibitory agents included anti-inflammatory drugs, sedatives, estrogens, antibiotics, organophosphates, chlorophenols, chlorinated hydrocarbons, and chlorinated cyclodienes. Braun and associates (*3*) observed 14 false negatives on testing 74 chemicals that caused terata *in vivo* and seven false positives on testing 28 compounds shown not to be teratogenic *in vivo*. Several classes of compounds, such as DNA damaging agents; inhibitors of macromolecular synthesis; and specific cell-surface receptors such as dexamethasone, hydrocortisone, isoproterenol, and vasopressin, cannot be characterized by this system. As a result, complementary tests would be essential for a teratogenicity screening system. Braun and Horowicz (*2*) have suggested that most of the false negatives generated by the attachment assay inhibit mesenchymal cell growth. Therefore, the palatal mesenchymal cell growth assay developed by Pratt et al. (*5*) could be used in combination with the cell attachment assay to predict teratogenic potential.

Since the *in vitro* test system cannot mimic maternal influences, the possibility has been discussed of combining the use of serum, urine, or amniotic fluid with test substances to more closely approximate the natural state. The effects of teratogens have been found to be dose-dependent within the assay system, although in some cases the concentrations required to inhibit cell attachment were substantially higher than those required to cause an embryonic malformation *in vivo*.

The attachment inhibition assay is a relatively simple and very rapid test, taking less than 1 hr to complete. Major equipment requirements include a liquid scintillation counter and an incubator. Disposable supplies such as concanavalin A, commercially available medium, ^{3}H-thymidine, pipets, and plastic bags for the disks represent a cost of approximately $20 to $50 per compound tested. One full-time technician could perform the assay, and since the test takes only 1 hr to perform, contamination problems often associated with longer term tissue culture work are eliminated. The assay system is now being validated by Microbiological Associates (Bethesda, MD).

Inhibition of Intercellular Communication in V79 Chinese Hamster Cells and Human Embryonal Palate Mesenchyme (HEPM) Cells

Introduction

The normal development of embryonic tissues and organs is thought to be partly dependent upon intercellular communications. Wilson (*6*) suggested that one of the mechanisms operating in teratogenesis was the alteration of cell membrane properties, leading to a failure in cell-cell interactions. Interference with any of the molecular structures involved in this type of communication, such as receptors, effectors, gap junctions, proteins, or microtubules, could conceivably affect the development of an embryo. A considerable amount of work has been done on the inhibition of cell-to-cell communications by tumor promoters. Trosko et al. (*7*) have postulated that the disruption of communication could be responsible for both tumor promotion and teratogenesis.

Yotti and associates (*8*) have developed a system based on the phenomenon of metabolic cooperation, which is a form of cell-cell communication characterized by the exchange of molecules between cells through permeable gap junctions formed at the sites of cell contact (*9*).

Methodology

The cells used in this assay are wild-type Chinese hamster V79 cells, generated from the lung of a normal animal, that are sensitive to 6-thioguanine (6-TGs) and X-ray-induced mutant cells that are resistant to 6-thioguanine (6-TGr). Cells are maintained in modified Eagle's medium supplemented with a 50% increase in concentration of vitamins, a 100% increase in nonessential amino acids, and a 50% increase in essential amino acids. The medium also contains 1 mM sodium pyruvate and 5% FCS. The cells are grown in petri dishes and maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air (*10*). The assay involves co-culturing a number of mutant V79 cells lacking the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) with wild-type cells that possess the enzyme. The enzyme-deficient cells ($HGPRT^-$) cannot metabolize 6-TG and will replicate in tissue culture when cultured alone. The wild-type population of cells has the HGPRT enzyme ($HGPRT^+$) and will metabolize 6-TG to a toxic substance that kills the cells. When $HGPRT^-$ (6-TGr) cells are co-cultured with $HGPRT^+$ (6-TGs) cells, the toxic metabolite is transferred from the 6-TGs to the 6-TGr cells and kills some or all of the $HGPRT^-$ mutants.

The number of cells killed depends upon the density of 6-TGs cells, since there must be cell-cell contact via gap junctions for the transfer to take place. Therefore, if addition of a test chemical to the medium causes a breakdown in cell attachment, the cultures will continue to grow. If there is no inhibition of cell attachment, the population of $HGPRT^-$ cells will decrease because of the transfer of the toxic metabolite from the $HGPRT^-$ cells to the $HGPRT^+$ cells.

A negative control for the assay is prepared in the absence of the test compound by co-culturing 400,000 to 500,000 6-TGs cells with 100 6-TGr cells in the presence of 10 μg of 6-TG/mL of culture medium. The noncytotoxic range must be established for each compound tested before the assay can be performed.

According to the procedure described by Welsch and Stedman (*10*), culture dishes (10 plates/concentration) are seeded with the two cell types. Four hours later, the test agent and 6-TG are added. After 72 hr, the culture medium is replaced with fresh medium containing only 6-TG. Cultures are terminated after the third or fourth day. The cells are washed with saline, fixed with 95% ethanol, stained with crystal violet, and the number of cell colonies is recorded. The endpoint of the assay is the ability of a compound to block metabolic cooperation, measured by its capacity to increase the number of cell colonies recovered after 7 or 8 days over the number of colonies found in the negative control plates. The results of the assay are considered positive only if the concentration resulting in the rescue of 6-TGr cells is not cytotoxic. To account for spontaneous mutations or mutations caused by the test substance making the cells resistant to 6-TG, 6-TGs cells are cultured in control medium or in medium containing the highest concentration of the test agent used.

Critical Review

Trosko and co-workers (*7*) found that a variety of chemicals have the ability to increase the recovery of mutant 6-TGr cells when co-cultured with wild-type 6-TGs cells. Positive responses in the assay included several chemicals known or suspected to be structural or behavioral teratogens (phenobarbital, 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), polybrominated biphenyls, polychlorinated biphenyls, mirex, Kepone, Dilantin, and Valium). Thalidomide was not positive in this system. Welsch and Stedman (*10*) noted that of six chemicals known to be teratogenic *in vivo* (TAP, 2,4,5,2′,4′,5′-hexabromobiphenyl, diphenylhydantoin, warfarin, ethylene glycol monomethyl ether, and ethylene glycol monoethyl ether) all inhibited metabolic cooperation in a dose-related manner at concentrations which were similar to calculated *in vivo* dosages. Two chemicals not known to have teratogenic potential *in vivo* (saccharin and L-ascorbic acid) had no effect on metabolic cooperation.

The assay at this time does not have a metabolic activation system. Biochemical parameters can be measured, and results are reproducible. Most chemicals that are water or solvent soluble may be tested in this system. Required equipment includes a laminar flow hood, water-jacketed CO_2 incubator, and routine tissue culture supplies. One technician with experience in tissue culture and sterile technique can operate the system.

The major limitation inherent in this system, as in many other *in vitro* teratology assays, is that the test looks at only one possible mechanism of action and therefore cannot be considered a broad prescreen for potential teratogens.

In an attempt to make this system more relevant, Welsch et al. (*11*) have recently used HEPM cells to study intercellular communication. The transfer of [^{3}H]-uridine between labeled and unlabeled HEPM cells is used as a measure of cell-cell communication. In this system, HEPM donor cells are placed onto coverslips which are placed into multiwell tissue culture plates. The cells are allowed to attach and then are pulse labeled with [^{3}H]-uridine for 3 hr. The cells are then cocultured with unlabeled cells for 3 hr with or without the test chemical to allow transfer of [^{3}H]-uridine nucleotides and incorporation into RNA. Cultures are washed and fixed with formalin at 4°C. The cells are then treated with warm (37°C) 5% trichloroacetic acid (TCA) twice for 5-min periods and finally rinsed with distilled water. The coverslips are then dried, attached to slides, and processed for autoradiography. Donor cells are identified by the high density of silver grains over the cells. Quantitative autoradiography is performed using an Artek 800 automatic grain counter. Radioactivity is detectable in recipient cells which are in direct contact with donor cells and in recipients making indirect contact with donors via other recipients. Recipient cells not making contact with donor cells do not have counts that exceed background.

Quantitative morphometric analysis has shown that the amount of intercellular communication is dependent on the number of gap junctions present. Only two chemicals have been tested using this system. The teratogen TPA inhibited transfer of [^{3}H]-uridine from donor to recipient cells but its structural analogue, 4α-phorbol-12,13-didecanoate, did not.

Cultured Mesenchymal Cells from the Human Embryonic Palate

Introduction

A considerable amount of work has been done on development of the secondary palate in mammals. It has been established that rodent and human secondary palates develop quite similarly, even though human palate development occurs at an earlier time in gestation. The cellular events occurring during development of the palate, such as growth, adhesion, death, and epithelial-mesenchymal interactions, and the sensitivity of palate cells to a variety of teratogens have made this process of interest to teratologists as a possible screen for teratogens (*5*).

Palates have been used in organ culture for some

time. Recently, a new technique has been made available that eliminates costly histologic preparations. Yoneda and Pratt (*12*), in collaboration with the American Type Culture Collection, have established the HEPM cell line. These mesenchymal cells originated from a day 55 human abortus at the time of palatal shelf elevation and before epithelial contact. This cell line has been used by Pratt and Willis (*13*) in a teratogen screening assay which evaluates growth inhibition in HEPM cells.

Methodology

Cells for the assay are maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM), which contains 20% FCS and 10% dimethyl sulfoxide (DMSO). HEPM cells are plated in 35-mm Falcon tissue culture dishes at a density of 4 to 5 $\times$ 10^4 cells per dish in 2 mL DMEM containing glutamine, penicillin G, streptomycin sulfate, and 10% FCS. Cultures are incubated in humidified air containing 7% CO_2 for 24 hr, at which time the medium is replaced with or without the test compound. The HEPM cells are then maintained in culture for an additional 72 hr. Test compounds are dissolved in medium, water, ethanol, or DMSO. A range-finding study is first performed to determine the concentration which inhibits cell growth short of toxicity and the concentration which inhibits cell growth by only 10 to 15%. The IC_{50} (concentration that inhibits cell growth by 50%) is determined using the mean from three to four dishes. The experiment is terminated after 96 hr, and cells are washed in calcium/magnesium-free phosphate-buffered saline and are detached in 0.05% trypsin/0.02% EDTA. Cell numbers are determined using a Coulter Counter, and the net growth is then calculated. Percentages are plotted against test chemical concentrations, and an IC_{50} for growth inhibition is interpolated. Since nonspecific growth inhibition does occur at high concentrations, 1 mM has been set as the concentration below which specific teratogenic inhibition of growth occurs.

Critical Review

The mesenchymal cell assay can reflect dose-dependent responses, such as the growth inhibition observed with the glucocorticoid dexamethasone (*12*). A microsomal, S-9, or whole cell metabolizing system can be incorporated into the HEPM cell cultures either directly or in dialysis bags (*14*).

Evaluation of the HEPM system by Pratt and Willis (*13*) focused on the list of chemicals and drugs tested by Braun and co-workers in the Braun mouse ovarian tumor cell-attachment assy (MOT). A total of 55 chemicals were tested. Of the 35 teratogens tested, 23 were inhibitory, and 12 of the nonteratogens were not inhibitory. When the results of the HEPM assay and the MOT assay are combined, they appear to be very complementary, yielding an overall predictability of 90%, with a false negative rate of 3%. The effective overlapping of these two screens demonstrates the advantages of combining several complementary *in vitro* tests to decrease the chances that an agent that is teratologically active *in vivo* will not be detected in a single screening system. The assay is presently being validated by the National Toxicology Program.

To perform the test, a laboratory must be equipped with a laminar flow hood, one or two tissue culture incubators, a phase contrast microscope, electronic cell counter, medium, and common glassware. The cost per HEPM assay is approximately $200 per compound. One or two technicians with experience in tissue culture work would be required to perform the assay. Pratt et al. (*5*) have indicated that HEPM cells are quite similar to *in vivo* palatal mesenchyme cells, and because of their human origin could be relevant as a screen for teratogens.

Porcine Granulosa Cell Culture

Introduction

Haney et al. (*15*) have developed a system that utilizes ovarian granulosa cells to screen chemicals for potential reproductive toxicity. Granulosa cells, located within the follicle and surrounding the ovum, produce the hormone progesterone, which can be quantitated using radioimmunoassay (RIA) techniques.

Methodology

Porcine ovaries containing highly differentiated preovulatory follicles are collected under sterile conditions and placed in buffered saline containing penicillin, streptomycin, mycostatin, and fungizone. Granulosa cells are obtained by Haney's procedure (*16*) of bisecting each follicle using sterile technique, removing the follicle lining from its base, and suspending it in a mixture of Ham's F-12 and DMEM containing penicillin and streptomycin. Care must be taken to excise comparable size follicles (6–9 mm), since the amount of hormone produced is directly related to the follicle diameter. The follicle linings are agitated and abraded between a glass rod and the wall of a plastic test tube, thereby suspending the granulosa cells in the culture medium. Debris from the follicle walls are removed by filtration through a nylon mesh filter. The cell suspension is then centrifuged and resuspended, and cell viability is estimated by vital staining with trypan blue using a hemocytometer. Basal progesterone production levels are determined for the population of cells, after which they are maintained for 24 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of this culture period, the cultures are aspirated and frozen at -196°C until use. Haney et al. (*15*) observed that the viability of the cells decreased only slightly in culture after cryopreservation. Aliquots of cells are thawed as needed. The cells are washed twice, resuspended, and checked for cell viability. The remaining cells are diluted with Ham's F-12 and DMEM containing 5% FCS and insulin.

The assay consists of four replicates for each group in multiwell culture plates, with treatment groups containing a vehicle (ethanol) and the test substance in concentrations from 10^{-8} to 10^{-5} M. Control cultures are treated with ethanol alone. Progesterone concentrations within the cells are determined by RIA (*17*). Estradiol has been used by Haney et al. (*15*) as a reference treatment to verify the responsiveness of the system, as its suppression of progesterone production in granulosa cells (up to 75%) is well documented (*16*). Additional biochemical parameters which could be tested in the future include response to luteinizing hormone/human chorionic gonadotropin, aromatase activity, or hormone receptor kinetics.

Critical Review

Since this assay is relatively new, few chemicals have been tested, and therefore, validation is still necessary. Water or solvent soluble chemicals may be tested in this system. Since reproductive toxicants may act by a variety of mechanisms other than cytotoxicity, Haney et al. (*15*) ran cycloheximide as an example of an inhibitor of protein synthesis. Results showed a decrease in progesterone production by 40% as compared to the control. Two isomers of DDT also tested positively in the assay. The insecticides malathion, parathion, and dieldrin, and the fungicide hexachlorobenzene all gave negative responses. However, due to the specificity of the test, a negative response by a chemical in the assay does not absolutely preclude the presence of reproductive toxicity. Although not readily available, human granulosa cells may be obtained as surgical specimens and used to confirm results with porcine cells.

Results from this test are reproducible and are capable of showing a dose-related response. The mechanism of action of the chemicals tested to date has not been established, and there is no indication in the literature that a metabolic activation system has been developed for the assay. The fact that the techniques involved in this procedure require only modest amounts of laboratory equipment and that materials can be obtained inexpensively through slaughterhouses makes the assay economically feasible. Technicians are required to have basic skills in cell culture and steroid RIA techniques.

Differentiating Embryonic Neural Crest Cells in Culture

Introduction

Embryonic neural crest cells have been used in culture (*18,19*) to test teratogenic and nonteratogenic compounds. These ectodermal cells have been shown to differentiate into autonomic neurons, glia, Schwann cells, and pigment cells. Cranial neural crest cells also differentiate into cartilage, bone, and connective tissue. Greenberg (*18,19*) found that cranial neural crest cells from 1.5-day-old chick embryos are multipotential and differentiate within 6 days to form pigment cells when cultured in FCS or neuronlike cells in horse serum. The cultured cells also synthesize melanin and an extracellular matrix containing collagen and glycosaminoglycans. During differentiation these cells are thought to respond to teratogens that alter cell-substrate interactions, mitosis, hormonal inductive factors, synthesis of gene products, and the elaboration of an extracellular matrix.

Methodology

According to Greenberg (*18,19*), primary cultures of neural crest cells are established by cutting windows into the eggs of White Leghorn chicks to find embryos whose cranial folds are in the process of fusing (stage 9). The vitelline membrane is peeled away, and the neural tube is removed and placed in a plastic Petri dish with Eagle's MEM containing 2% chick embryo extract, 5% horse serum, and antibiotics. Over a 2-day period, the neural tubes will attach to the dish, and cells of neural crest origin will migrate away from the neural tubes on the surface of the culture dish. The neural tubes are removed at the end of the 2 days, and the remaining monolayer of neural crest cells is suspended and distributed to 35-mm culture dishes. Compounds to be tested are added to the cells in culture 1 day later. Cultures are examined daily for approximately 1 week by phase contrast microscopy to assess growth and differentiation. Each assay is run twice with four dose levels and a control. Any compound causing cell detachment, a lower cell density, changes in cell shape, or lack of differentiation compared to untreated controls is considered to have a positive effect on the culture. The minimum effective concentration that could induce one of these effects is considered to be the end point of the assay. It is also possible to assay the cells biochemically on day 7 for choline acetyltransferase activity or melanin synthesis.

Critical Review

When metabolic activation has been necessary, the test compound has been incubated with the post-mitochondrial supernatant fraction (S-9) of liver microsomes of phenobarbital-treated rats plus an NADPH-generating system. Since the S-9 mixture was found to be toxic when added directly to the cell cultures, Greenberg (*19*) combined the fraction with the test compound in a dialysis bag and placed it in the culture medium. Over a 4 hr period, the compound and its metabolites diffused through the culture medium.

The effects of chemicals tested within this system are dose-dependent. Nine of eleven compounds previously shown to be teratogenic *in vivo* induced detectable morphologic alterations in the neural crest cells. Phosphoramide mustard, norchlorocyclizine, diphenylhydantoin, and β-aminopropionitrile elicited positive responses in the assay at concentrations similar to maternal blood levels associated with malformations *in vivo*. In con-

trast, 5-bromo-2'-deoxyuridine and retinoic acid were active at lower concentrations in culture than *in vivo*. It has been postulated that maternal protective processes are at work *in vivo* to decrease exposure to the embryo. Thalidomide and acetylsalicylic acid had no effect on neural crest cultures.

Proponents of this system feel that neural crest cells are a relevant model for the whole animal because the cells proliferate and form cellular associations, adhere to the substrate, produce extracellular matrix, and differentiate. However, cultures such as these do not possess the cell interactions that can be found in the more complex systems of organs or whole embryo culture where many cell types are found to interact. Homotypic cultures such as the one described here can provide a quantitative and qualitative assessment of test compounds more easily and faster than complex systems, and the biochemical determinations of melanin synthesis and choline acetyltransferase activity are reproducible. One of the major disadvantages in this type of system is that biochemical assays require larger numbers of cells than morphological determinations. Greenberg (*19*) has stated that typically 6 to 9 dozen eggs will produce enough neural crest cells from dissected embryos to perform duplicate tests using morphological end points on six concentrations of two compounds. Therefore, teratology screens which use primary cell cultures such as this system are limited by the number of cells that can be generated.

The assay is labor-intensive because of the difficulty of the dissections. However, one skilled technician with tissue culture experience and training in the dissection technique could perform the assay. Required laboratory equipment includes a laminar flow hood, an incubator for the cell cultures, an egg incubator for the chick embryos, a phase contrast microscope, and disposable tissue culture supplies.

Limb Bud Mesenchyme Cell Culture

Introduction

Kochhar (*20*) and Hascall et al. (*21*) have shown that cell to extracellular matrix interactions are essential for normal embryonic development. A system that has the ability to detect disruption in these relationships can provide relevant data about the effect of teratogens on morphogenesis. The extensive use of limb bud (LB) cell culture in the investigation of the processes involved in chondrogenesis has led to the development of an *in vitro* screen for teratogens. Wilk et al. (*14*) have described LB mesenchyme cells that grow and differentiate into chondrocytes in culture and synthesize an easily quantifiable extracellular matrix product, namely, sulfated proteoglycan. The amount of proteoglycan produced depends on the extent of growth and differentiation over a 6-day culture period. Agents that interfere with growth or differentiation reduce the amount of proteoglycan present in the chondrocytes.

Methodology

According to Hassell et al. (*22,23*) and Wilk et al. (*14*), LBs are isolated from 10- to 12-day-old mouse embryos or day 4 chick embryos, and then are dissociated by trypsinization, suspended in MEM supplemented with fetal bovine solution (FBS), vortexed, and filtered to remove debris. The resulting single-cell suspension is used to seed individual cultures in 24-well Costar dishes, which are placed in a humidified CO_2 incubator at 37°C for 1 to 3 hr to allow cell attachment. Once the cells attach, wells are flooded with either CMRL culture medium containing 10% FBS (mouse cultures) or Eagle's MEM with 10% FBS (chick cultures). On the following day, the test compound dissolved in water, ethanol, DMSO, or control solvents are added to the cells via the medium, which is changed on days 1, 3, and 5. The total treatment time is designed to cover the critical period of LB cell differentiation.

The cultures are monitored daily by phase contrast microscopy to follow cell differentiation. The accumulation of sulfated proteoglycans is determined on day 6 after the cells are stained with alcian blue (*23*). Unbound dye is removed from the cells by several acid rinses. After the bound dye is extracted from the cells, it may be quantitated spectrophotometrically at 600 nm. Normal cells in this system show a progressive increase in staining, which plateaus between days 5 and 6. A positive effect by a teratogen in this system would elicit a decrease in proteoglycan production. Cellular and extracellular macromolecular synthesis may be quantitated by the incorporation of radiolabeled compounds and scintillation counting. Additional effects that have been observed include cell detachment from the substratum, decreased cell proliferation, cell morphology change, and failure to differentiate.

During the culture period, cell number increases four- to sixfold, and gene products change. Compounds that act by inhibiting cell proliferation test positively in this system, since a reduction in cell number would mean a corresponding decrease in extracellular matrix. Cell products change from type I to type II collagen and from mesenchyme proteoglycan to cartilage proteoglycan, and the synthesis of fibronectin is terminated. Any compound that interferes with these processes could be considered a potential teratogen. The mechanism of chondrogenesis inhibition can be determined by radioisotope incorporation or chemical measurement.

Tests are conducted in triplicate; a range of doses is used for each compound. An equivalent amount of vehicle is used in the control cultures without the test agent. The amount of Alcian blue staining at each dose is expressed as a percent of the control cultures. The teratogenic potential of a compound is quantitated by finding the concentration required to reduce staining by 50% (TP_{50}) from a dose-response curve that may be correlated to proteoglycan production.

Critical Review

Some chemicals found to be inhibitors of chondrogenesis in this assay are cytosine arabinoside, actinomycin

D, vitamin A, and hexachlorophene. Proven and suspected teratogens such as β-aminopropionitrile and diphenylhydantoin have also been tested with positive results. Concentrations of the chemicals tested thus far have been in the range of blood levels known to cause malformations *in vivo*. Thalidomide tested negatively in the assay without the use of an activation system. Nonteratogenic chemicals had no effect on growth and differentiation.

This system may be used in conjunction with one of several metabolic activating systems. Serum from treated animals or humans may be added to the culture medium to test for teratogenic metabolites produced *in vivo*, or a combination of an S-9 fraction and NADPH-generating system can be used to activate compounds. In addition, Hassell and co-workers (*23*) are attempting to coculture primate liver cells with the LB cells.

The assay requires one or two technicians with knowledge in basic animal care, surgery, liquid scintillation counting, and tissue culture techniques. Equipment requirements include a laminar flow unit, incubators, liquid scintillation counter, microtiter dishes, and time-pregnant mice.

Litton Bionetics currently offers the assay as a broad pre-screen for teratogens. The procedure described by Guntakatta et al. (*24*) is a modified method which uses radiochemicals for the assessment of chemical effects on sulfated proteoglycan and DNA synthesis in mouse LB cells. In this method, hindlimbs are dissected from 10 to 15 dams and pooled in 4°C PBS. The LBs are dissociated with trypsin-EDTA, and the resulting cell suspension is filtered, washed, and resuspended in CRML 1066 culture medium containing L-glutamine, penicillin, streptomycin, and heat-inactivated FBS. In routine cultures, 10-μL aliquots of the cell suspension are seeded into 16-mm wells of 24-well culture dishes. The cells are allowed to attach over a 2-hr period and then are fed with 1.0 mL CMRL medium and incubated at 37°C in 95% air and 5% CO_2 in a humidified atmosphere.

The test chemicals are added to each spot 24 hr later and control cultures are re-fed with culture medium. After 72 hr of treatment, the medium is removed and the cultures are re-fed with reduced sulfate labeling medium containing [^{3}H]-thymidine, $^{35}SO_4^{-2}$, and test chemical, and incubation is continued for another 24 hr. Spot cultures are washed with phosphate-buffered saline and macromolecules precipitated with 10% TCA. After 18 to 24 hr at 4°C, the precipitates are washed with cold TCA and then solubilized in 1% sodium dodecyl sulfate (SDS) at 37°C. After 4 to 8 hr, the radioprecursors in the SDS-solubilized material are estimated in a liquid scintillation counter. The accumulated cartilage proteoglycans are estimated by Alcian blue staining. Cultures are stained with 0.5% Alcian blue in 3% glacial acetic acid. After 2 hr, the cultures are treated with 3% acetic acid for 10 min and then 2 min with glacial acetic acid. Bound stain is eluted for 16 to 24 hr with 8 *M* guanidine hydrochloride at 4 to 6°C. The absorption of Alcian blue dye in the spot is then determined at 600 nm. The inhibition of either proteoglycan synthesis or DNA synthesis was determined for 19 of 22 known mouse teratogens by Guntakatta et al. (*24*) using double-labeling experiments. Five nonteratogens tested negatively in this assay. The overall accuracy of the system was 89% with a false negative rate of 14.8%. No false positives were observed. Development of an exogenous activating system for the mouse embryo LB cell culture system is currently under way.

Differentiation of Rat Embryo Midbrain and Limb Bud Cells in Culture

Introduction

Flint recently developed a short-term assay for teratogens using differentiating rat embryo midbrain (CNS) and limb bud (LB) cells in culture. Two methods using both *in utero* chemical exposure and *in vitro* chemical exposure are described in the literature (*25,26*). Most of the *in vitro* teratology screening systems developed to date require the addition of the test substance directly to the culture medium, with or without the use of a metabolic activating system. Occasionally, serum from humans or amniotic fluid from pregnant animals exposed to teratogens has been added to the medium of cultured cells. In early work by Flint et al. (*25*), rat embryos were exposed to a variety of teratogens and nonteratogens *in utero*, and embryonic LB cells and midbrain cells were subsequently cultured. This approach allows for maternal metabolism of the test agent and gives the yolk sac and placenta an opportunity to exert an influence on the compound. The cells are cultured for 5 days, permitting cell differentiation. During this time, cells in LB cultures group together in small foci of chondrocytes and secrete sulfated proteoglycans. CNS cells differentiate into neurons, which are organized into foci surrounded by fibroblasts. The growth of the cells is determined by total protein and differentiation by morphometric assay and the incorporation of radiolabeled substrate. In a more recent method, the test compound is added directly to the culture medium, which simplifies the procedure and conserves animals.

Methodology

In Flint's *in vivo* exposure procedure (*25*), the test compound or vehicle control is administered intraperitoneally to pregnant rats on day 12 of gestation. Doses normally range approximately midway between the highest and lowest active dose for teratogens and at the maximum nontoxic acute dose for nonteratogens. The animals are sacrificed 16 hr after dosing, and embryos (34–36 somites) are removed aseptically to a medium containing horse serum and Earle's Balanced Salt Solution at 37°C. LB cells are prepared by the methods described by Ahrens and co-workers (*27*), and CNS cells

are prepared from the mesencephalon by techniques developed by Flint (*28*). Occasionally, embryonic tissue from a pair of animals will have to be pooled to provide enough tissue to complete an experiment. Flint et al. (*25*) have justified this procedure by demonstrating that there is little variation between animals for the parameters noted. Briefly, tissues are dissociated by trypsinization and resuspended in Ham's F12 culture medium containing FCS, L-glutamine, penicillin, and streptomycin. Aliquots of the two cell types are delivered to plastic dishes for 2 hr at 37°C to allow for cell attachment. Culture medium (2 mL) is then added to each dish, and cultures are incubated for 5 days at 37°C in an atmosphere of 5% CO_2 in air and 100% humidity. Within each dish, a small circular island of cells forms. One LB cell island and two CNS cell islands are cultured in each dish for protein content and isotope incorporation. Five CNS and three LB cell islands are cultured in each dish for determinations of cell differentiation. Five replicate dishes are prepared for each assay. LB cultures are fixed in 10% formaldehyde, and proteoglycans are stained with alcian blue. CNS cultures are fixed in 10% formaldehyde and stained with hematoxylin. Protein content is estimated by the method of Lowry et al. (*29*). Differentiation is measured by the number of stainable foci present and ^{3}H-γ-aminobutyric acid (H-GABA) incorporation in CNS cells and [^{35}S]-sulfate incorporation in LB cells. H-GABA and [^{35}S]-sulfate are determined by exposing CNS cells to H-GABA for 1 hr on day 5 and LB cells to [^{35}S]-sulfate for the entire day 5 culture period. At the end of the exposure period, radioactivity is determined with a liquid scintillation counter. Differentiated cells are analyzed morphometrically with a dissecting microscope attached to an Artek 980 colony counter that automatically counts areas of high contrast (darkly staining foci of cells).

In the wholly *in vitro* procedure, embryos are removed and dissected, and the cells prepared as described in the first method. An S-9 rat liver metabolizing system may be constituted according to the procedure described by Ames et al. (*30*). The prepared dishes of cells are incubated at 37°C in 5% CO_2 in air and 100% humidity for 2 hr, after which the test compound with or without the S-9 mix is added in a final volume of 2-mL culture medium. Cultures are exposed to the compound at concentrations of 0.1, 1, 10, 100, 200, and 500 μg/mL. If used, the S-9 mix is removed after 2 hr due to cytotoxicity and replaced with fresh culture medium. Concurrent control cultures receive the appropriate volume of solvent only. Cells once again develop distinct micromass islands during the 5 days in culture. At the end of this period, CNS cells are fixed with 10% aqueous formaldehyde and LB cells with aqueous cetylpyridinum chloride/10% formaldehyde. CNS cultures are stained with Delafield's hematoxylin and LB cultures with alcian blue. The number of stained foci is measured automatically by an Artek 980 colony counter. The concentration at which each compound inhibits the formation of differentiated foci by 50% of the control value (IC_{50}) is determined. Inhibition of differentiation in either CNS or LB culture is used as the criterion for potential teratogenicity.

Critical Review

Nonteratogens had no effect on cultured differentiating rat embryo cells in the *in vivo* exposure assay. Teratogens inhibited LB and CNS cultures by 44 to 70% of the controls. Azathioprene, dexamethasone, 2,4-dichlorophenoxyacetic acid, ergotamine tartrate, mitomycin C, phenobarbitone, and methyl testosterone all tested positively by inhibiting growth and differentiation. No nonteratogen was found to inhibit culture development by more than 20%. Of the teratogens, amaranth, EDTA, griseofulvin, and thalidomide inhibited development by less than 20%. However, by employing discriminant analysis of the data, only nitrilotriacetic acid could be identified as a false positive and amaranth as a false negative. Overall sensitivity of the assay was 92% correct for teratogens and 94% correct for nonteratogens in a total of 31 compounds.

In the *in vivo* exposure method, repeat experiments using two known teratogens, aldrin and diphenylhydantoin, showed that interexperimental variability was low. The assay was able to differentiate between the teratogenic activity of several pairs of compounds with similar chemical structures. The sensitivity of the assay was 93% for prediction of teratogens, and the specificity was 89% for prediction of nonteratogens in a total of 46 compounds tested (27 teratogens, 19 nonteratogens). If inhibition of differentiation of only one cell type was considered, then the predictive value was lower (CNS 85%, LB 82%). False negative compounds were 2,4-dichlorophenoxyacetic acid and thalidomide. The false positive compounds were glutethimide and dimenhydrinate.

These assays are relatively short term (5 days for culture development and 1 day for quantification), use small numbers of rats, and exhibit dose-related responses. The *in vivo* assay excludes the possibility of nonspecific cytotoxicity generated by direct addition of test compounds to the culture medium. Both assays appear to have the same predictive capabilities.

Required equipment for the assays include a liquid scintillation counter, incubators, microscope, radioisotopes, and routine tissue culture supplies. The value of both assays as a screen for teratogens will have to be evaluated after additional chemicals are tested.

Drosophila Embryonic Cell Culture

Introduction

Bournias-Vardiabasis and Teplitz (*31*) have developed an *in vitro* teratology screen using *Drosophila melanogaster*. The assay is based on a quantitative determination of muscle and neuron differentiation in embryonic cell cultures. Early work by Seecof and Donady (*32*) showed that primary cell cultures of Drosophila provided a very effective system for the study of embryonic

stem cells that differentiate into neurons and muscle cells. They noted in their studies that differentiation of neuron and muscle *in vitro* was very similar to that *in vivo*, with only a short time lag in the cultured system. It is assumed that Drosophila embryo muscles and neurons are derived from the same basic mechanisms that operate in vertebrates and that the cells respond to chemical insult in the same way as vertebrate cells. Bournias-Vardiabasis and Teplitz (*31*) demonstrated that Drosophila cells did, in fact, respond very similarly to vertebrate cells when exposed to insulin and various metabolic inhibitors.

Methodology

The basic procedure as described by Bournias-Vardiabasis et al. (*31,33*) involves the mass collection of Oregon R strain Drosophila eggs over a 2-hr period using population cages. The eggs are sterilized at the early gastrula stage, before any morphological or ultrastructural differentiation. Eggs are then homogenized and centrifuged so that a pellet of embryonic cells is collected. The cells are plated in 35-mm culture dishes, and Schneider's medium containing 18% heat-inactivated FCS is added. Once the cells attach to the bottom of the dish (15–20 min), the medium is replaced with medium in which the test compound is dissolved. Agents may be dissolved in either water, alcohol, or DMSO. All solvents have been tested and allow normal cell differentiation. The proper range of concentrations to be tested is determined by feeding the compound to the adult female Drosophila and determining the LD_{50}. The concentrations used in the assay would then be a 10- to 100-fold dilution of this dose. Controls are treated in the same manner as experimental compounds. After the test agent has been added, the cultures are placed in 25°C incubators and are scored for cell and tissue differentiation 24 hr later. During this period, several types of cells differentiate from their stem cells, as observed by phase contrast microscopy. Neuroblasts divide, forming daughter neurons, which in turn form small ganglia; and myoblasts divide and become mononucleated myocytes, which eventually become multinucleated myotubes. The differentiated cultures are rinsed in 0.1% trypsin in saline solution to remove cellular debris, fixed in Zenker's solution, stained with hematoxylin, and counterstained with Evans' blue. If interference with normal differentiation occurred at an early stage of development, the number of cells should be reduced. Interference later in development could result in abnormal myotubes.

In addition to the number of ganglia and myotubes present, axons, F cells, C cells, pulsations, and neuromuscular contacts may be counted. Biochemical parameters can also be measured, such as the production of acetylcholinesterase or choline acetyltransferase. However, to keep the assay simple, endpoints are usually limited to counts of myotubes and ganglia. The speed of the assay can be increased by the use of an image analyzer (Bausch and Lomb Omicon), which automatically scores the number of myotubes and ganglia, thus decreasing the number of hours required to perform the test. Four dishes are scored for each chemical tested, and the number of myotubes and ganglia are averaged and computed as a percentage of the control average. The assay provides dose-response relationships for ganglia and myotube differentiation. Three or more separate trials are run for each compound. To be classified as a teratogen, an agent must have caused a reduction in differentiation as evidenced by a 50% decrease in the number of neurons and/or muscle in treated versus control groups. The assay takes 3 days to complete.

Critical Review

There has been good correspondence between this assay and other systems such as *in vivo* animal studies and human epidemologic studies. In an attempt to validate the assay, Bournias-Vardiabasis et al. (*33*) tested over 100 commonly used industrial chemicals, drugs, and food additives. Of the 45 agents testing positively in the assay, only two false positives were found (*p*-aminobenzoic acid and dimethylacetamide). Fifty-five chemicals had no effect on embryonic differentiation, and only four of those chemicals could be classified as false negatives (pilocarpine, lead nitrate, ethyl alcohol, and cylophosphamide). Among the chemicals testing positively in the assay were acetylsalicylic acid, adenine, amethopterin, atropine, urethane, mercuric chloride, and testosterone. Thalidomide interfered with muscle and neuron differentiation without the use of a microsomal activating system, in contrast to previously published data which indicated that thalidomide's action was dependent upon metabolic activation.

Teratogenic chemicals have been activated in this system by several methods, including the use of serum from animals fed suspected teratogens, serum from patients on chemotherapy (vincristine and nitrogen mustard), and low concentrations of human urine, with positive results. Methods are currently being developed for the routine addition of an S-9 fraction or the microsomal fraction (S-27) from Drosophila (*34*).

This is an inexpensive assay for which a full-time technician could easily be trained. Tissue culture experience is not a prerequisite for performing the assay. Required equipment includes Drosophila population cages, Drosophila food, tissue culture incubator, homogenizer, glassware, and media. Total start-up costs are modest, excluding the cost of the image analyzer, which is not a necessity. The use of a specialized portable incubator can expand the capability of the system to testing a variety of gases. The assay must be conducted in a room that is isolated from mammalian tissue culture work because of the possibility of yeast contamination from the Drosophila food.

This assay, in addition to its use as a short-term screen for potential teratogens, also has the potential for investigations into the mechanisms of teratogenesis. Because of the vast amount of information on the genetic

characteristics of Drosophila, this system lends itself to the study of genes that control embryonic and fetal development. As with other cell culture assays, this system could be used in conjunction with other complementary tests.

Buzin and Bournias-Vardiabasis (*35*) have recently used two-dimensional gel electrophoresis to study protein synthesis in Drosophila embryonic cells treated with drugs. Dose-response studies have shown a correlation between the degree of inhibition of differentiation and the level of induction of three specific proteins. These proteins appear to be identical to three proteins which had previously been induced by heat shock. Nine teratogens have been tested individually in Drosophila primary embryonic cells, and the three proteins (hsp 23, 22a, and 22b) were induced in each case. Three teratogens and eight nonteratogens did not induce these proteins. Buzin and Bournias-Vardiabasis have stated that the chemical induction of these heat shock proteins may reflect specific types of stress which in themselves may be teratogenic (*35*).

Differentiating Neuroblastoma Cells

Introduction

Murine neuroblastoma cells (clone NIE-115) have recently been used by Mummery and co-workers (*36*) in an assay that detects agents capable of interfering with growth and differentiation. Neuroblastoma cells are precursors of sympathetic ganglia derived from malignant tumors originating from neural crest cells (*37*). Under appropriate conditions, the cells will undergo terminal neuron differentiation. Some of the characteristics exhibited by these differentiating cells include extension of neurites (processes longer than the diameter of the cell body), production of enzymes, and the development of an electrically excitable plasma membrane (*38*). While working with these cells, Schubert and associates (*39*) noticed that if serum was removed from the cells during culturing, the neuroblastoma cells could be induced to differentiate. Since neural tissue has been known to influence several processes of embryogenesis such as limb development, and neuronal damage has been implicated as a factor in teratogenesis, a screen for teratogenicity utilizing differentiating neural tissue could be of value.

Methodology

The procedure developed by Mummery et al. (*36*) entails growing mouse NIE-115 neuroblastoma cells in DMEM containing glucose and FCS. Subcultures of stock cells are plated in 1.6-cm diameter wells in 1 mL of DMEM containing 7.5% FCS. The cultures are incubated at 37°C for 24 hr, at which time the medium is changed, and the test substance, dissolved in water, ethanol, or DMSO, is added to the wells at various concentrations. Both the medium and the test substance are replaced on day 4. Cells grown in medium containing serum grow exponentially as rounded cells and are confluent by day 7. Control cultures at this time exhibit low levels of spontaneous differentiation in the form of neurite production. Any substance that induces the formation of neurites in significantly greater quantities than the control is considered to be positive in the assay. Viable cells are identified by trypan blue exclusion and are counted and expressed as a percentage of the control. Three parameters are measured in triplicate for each test substance: (1) the minimum dose for 100% cell death; (2) maximum nontoxic dose that causes no decrease in cell number; and (3) if applicable, the minimum dose resulting in an effect on differentiation (lowest effective dose). The cell number at the end of the test is also determined for the lowest effective dose for each substance and expressed as a percentage of the control.

Inhibition of differentiation is also determined in the assay by culturing cells in DMEM with FCS, but after 24 hr the medium is replaced without FCS. Test substances are added to the medium and replaced on day 4. By day 7, all control cells have differentiated, forming a network of thin neurites. Wells are photographed and scored positive if neurite formation is inhibited.

Critical Review

Mummery et al. (*36*) tested 42 teratogens and found that 32 induced morphological differentiation, including mercaptopurine, urethane, and hydroxyurea. Three known teratogens could not be categorized by the system. Thalidomide was relatively insoluble, leading to high concentrations of the solvent, and methylthiouracil and phenytoin both induced differentiation at toxic doses. Of the 19 nonteratogens tested, 14 gave negative results. Cyclamic acid could not be categorized because of the high concentrations required to produce an effect, and the remaining four scored positively. Only 3 of 57 chemicals tested by Mummery et al. inhibited differentiation (retinoic acid, insulin, and Hg^{2+}). It was also noted that the toxic dose for cells grown in the absence of FCS was approximately one order of magnitude lower than cells grown in the presence of FCS. Mummery et al. (*40*) had previously shown that serum tended to reduce the potency of toxic chemicals in exponentially growing neuroblastoma cells.

Overall, the assay system was capable of correctly identifying 49 of 57 (86%) teratogenic and nonteratogenic agents. Of the 39 teratogens, 4 were false negatives, and of the 18 nonteratogens (22%), 4 were false positives. The system has generated results similar to Braun's tumor cell attachment assay (*1,4*), with some improvement. Judging from the compounds tested, the system appears to be sensitive to teratogens that alter tissue development and inhibit growth processes. Metabolic activation was not employed in this assay, but the option of preincubation of agents with liver microsomes (*30*) has been discussed as a possibility. The use of embryonic neuroblastoma cells in a test system such as the one described could be of value in the study of

the regulatory role of the plasma membrane in growth and differentiation and in the mechanisms involved in teratogenesis.

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