Environmental Health Perspectives
Vol. 72, pp. 211-219, 1987

Teratological Research Using *In Vitro* Systems. II. Rodent Limb Bud Culture System

by Leonard Friedman*

This review represents a compilation of information related to the rodent limb bud culture system from approximately 80 publications after 1969 and conversations with workers in the field. Most of these papers and book chapters refer exclusively to studies with the mouse limb bud. Sections in this review include historical review, end point measurements, activating systems, types of compounds studied and dose response, reproducibility, statistical analysis, equipment and personnel requirements, mechanisms, and summary.

Introduction

The rationale for the development and study of *in vitro* limb bud systems, particularly mammalian limb bud systems, resides in the use of organ culture systems (*1*). Much information is available on the early development of limb buds in the embryos of reptiles (*2*), amphibians (*3*), and birds (*4–6*), but according to Merker (*7*), "a confirmation of these findings in mammals is still lacking as the technical difficulties of the intrauterine position, the surrounding fetal fluid, etc., are too great." The period of late organogenesis, during which time the limbs develop, cannot be fully studied in the whole embryo *in vitro*, apparently because of inaccessible nutritional requirements for the embryo in culture. Cell culture techniques do not permit study of the morphogenetic differentiation of limbs (*8*) similar to *in utero* differentiation. These processes, however, can be observed by organ culture techniques.

Strangeways and Fell (*9*) first reported successful *in vitro* culture of limb buds. Undifferentiated limb buds from fowl embryos were allowed to develop in plasma clot cultures for 7 days. Although the results were inconsistent, cartilage production took place in the explants, and the skeletogenous tissue frequently showed the early stages of development of normal limb skeleton.

Satisfactory techniques for the culture of mammalian limb buds were not developed for some time. An early attempt by Shepard and Bass (*10*) to grow rat limb buds in culture produced only partial success: although cartilage developed within the explants, specific rudiments could not be identified. Kochhar and Aydelotte (*11–13*) studied the effects of 1-azetidine-2-carboxylic acid on mouse embryo limb buds *in vitro*. Limb buds developed well under their culture conditions, and after 6 days in culture, recognizable elements of the limb skeleton chondrified and began to ossify. Many recent reviews on the development of methodology for mammalian limb culture for use in studying the effects of chemicals on these systems are now available (*7,8,14–23*).

With appropriate culture conditions, development of the limb *in vitro* will mimic the development *in vivo*, albeit at a somewhat slower rate. Although some distortions may appear, anlagen of the major bone elements appear in a clearly recognizable form in the cultured limb, and provide a useful technique in the study of embryogenesis (*22*). Experimental modification of whole embryos either *in vitro* (*23,24*) or *in utero* via maternal treatment (*20,25*) has also provided limb buds for study. The technique of treating the limb bud with test chemical *in vitro*, then grafting it onto the whole embryo, has also been explored (*6*). The degree of limb development (size and shape) is largely dependent on the age or stage (somite number) of the embryo from which the limb bud is removed; therefore, reproducibility of results demands careful standardization of both culture conditions and staging of the embryos (*8*). Mouse embryos of 11 to 13 days' gestational age and containing 40 to 55 somites are generally used for full development of the limb, including the bones of the paw (*14*).

Limb buds (usually forelimb) have been cultured from several species of animals, including chicken (*1,26*), mouse, rat, rabbit (*15,20*), marmoset (*27*), and even human [unpublished data cited by Rajan (*28*)]. Consistently reproducible results, however, have been obtained chiefly with limb buds from mouse embryos (*14*); thus, they have been used almost exclusively. Organ culture techniques have also been applied to embryonic

*Division of Toxicology, Food and Drug Administration, Washington, DC 20204.

bone and cartilage fragments from species such as calf (*29*), mouse (*30*), rat and human (*31*), and chicken (*32*). Finally, some investigators (discussed elsewhere) have studied the differentiation of limb bud cells in culture (*33–38*).

Methodology

Aydelotte and Kochhar (*13*) successfully adapted Trowell's technique (*39*) for cultivation of mammalian organs. Briefly, forelimb and hindlimb buds were dissected from mouse embryos on day 12 of pregnancy (34–42 pairs of somites). The explants were placed on ultrathin Millipore filters supported by stainless steel grids over a medium consisting of 75% Bigger's or BGJ medium supplemented with ascorbic acid, penicillin, streptomycin, and fetal bovine serum. Cultures were maintained in a humid atmosphere of 5% CO_2 in air at 38°C and placed on fresh medium every 3 days. The right limbs from each embryo served as controls, and the corresponding left limbs were designated as experimentals. Kochhar (*14*) has indicated that excellent results were obtained with only minor modification from Trowell's technique, namely, substitution of Nucleopore filters for the Millipore filters and the occasional use of homologous adult mouse serum in place of the fetal bovine (calf) serum. Lessmollmann et al. (*40*) demonstrated comparable growth and differentiation of mouse limb buds cultured in a completely chemically defined medium where a 25% salt solution was substituted for the serum and grown under conditions similar to those used by Aydelotte and Kochhar (*13*). In a more recent modification, Neubert and Barrach (*15*) substituted a submerged roller-bottle culture system for the stationary systems, using either the chemically defined or serum-supplemented media. This modification overcame the disadvantage (*7*) of the limb buds having to adhere to and grow on a filter and resulted in the production of more naturally appearing explants (*8*). In addition, the explant has greater accessibility to nutrients, gases, and drugs (*8,41*), but apparently requires a later embryonic stage (e.g., 43–45 somite stage) for satisfactory development of the forepaw skeleton (*15*). Various techniques have been used to prepare and fix the explant, depending on the morphological or biochemical endpoints of interest. The explant may be examined at different stages of development, but usually by the sixth day of culture, maximum growth and differentiation are evident (*15*). To prepare the limb for visualization, limbs are rinsed free of the medium; fixed in Bouin's fluid; stained with alcian blue, toluidine blue, or methylene blue (*42*); cleared with such materials as acetic acid (*15*), methyl salicylate (*43*), or xylene (*44*); and usually stored in cedar wood oil (*13*) for visualization with low power microscopy or microprojection (*10*). For histological examination, specimens are embedded, sectioned, and stained by standard techniques (*13,45*). According to Kochhar (personal communication), the tissue processing can take place *in situ* without removal of the limb from the filter in the stationary culture system.

Critical Review

Mammalian limb bud culture holds great promise for uncovering basic developmental processes that occur during late organogenesis and for elucidating modes of teratogenic action. Its usefulness in screening, however, is questionable at present, possibly because of the different methodological approaches, end points, methods of quantitation, types of chemicals examined, and standardization procedures (especially in regard to stage of fetus), and because of the presence or absence of activation systems.

End Points

The mammalian limb bud culture system permits not only differentiation at the cellular level (e.g., production of cartilage-producing cells from nondifferentiated precursor cells) but also morphogenetic differentiation in which the differentiated cells are arranged to form the anlagen of particular structures (e.g., discrete long bones of the limb). Upon growth, the resulting explant closely resembles that seen in the limb developed *in vivo*. Furthermore, the limb bud of the mouse limb develops along the proximodistal axis of the limb as in the whole embryo developing *in utero* (*7*). This complex process allows for the measurement of a large number of morphological, morphometric, and biochemical end points. Morphological criteria such as the overall appearance, size, and shape of the limb bud after culture for a designated period are used to assess the degree of normal development of the explant. Such observations allow for the demonstration of specific compound-induced malformations of the digits such as syndactyly and phocomelia (*46*), which mimic some of the types of abnormalities seen *in vivo*. The developing limb bud is evaluated in relation to the formed cartilage elements by various procedures. In early work, Aydelotte and Kochhar (*13*) photographed specimens or drew the outlines of the limb buds and associated cartilage elements with the aid of the camera lucida. The developing limb bud was described simply in qualitative or relative terms.

In an attempt to develop a semiquantitative method for evaluating effects of drugs, Neubert et al. (*47*) devised a scoring system in which a sum of points was given for ideal development of the whole limb in culture, each of the cartilaginous anlagen of the limb (scapula, humerus, ulna, radius, and carpalia), and the individual digits of the paw skeleton. This system permits statistical analysis of the data and identification of abnormal areas relative to control limbs; however, ratings are subjective and information about type of abnormality is lacking. Agnish and Kochhar (*24,48*) adopted a less subjective approach for quantifying the amount of cartilage in the cultured limb. The area occupied by each cartilage zone or unit was determined by projecting a photo-

graphic slide of the stained and cleared limb onto squared graph paper and counting the number of squares or area units delimited by the outline of each of the zones. [Shepard and Shiota (*49*), used a similar system but measured the projected surface area with a planimeter.] According to the authors, this method permits a separate, quantitative analysis of each of the limb components, as well as comparison of total limb area units among the different limbs. However, only two-dimensional (area) measurements are made of the region, and quantitative assessments of shapes are lacking. Kwasigroch et al. (*50*) used an automatic image analyzer to determine actual areas and shapes ("form factors") of the whole limb and bone anlagen in the *in vitro*-developed limb.

Since the formation of cartilage is associated with the biosynthesis of two tissue-specific macromolecules, type II collagen and sulfated proteoglycan(s), quantitation of these components can serve as biochemical markers of the differentiation process (*18*). Other biochemical end points are glycosaminoglycan metabolism (*51*), localization of collagen type (*52*), biosynthesis of collagen and prolyl hydroxylase activity (*17*), chondroitin sulfate synthesis (*53*), and hydroxyproline levels (*16*). Additional end points related to non-cell-type specific biochemical processes include DNA, RNA, and protein concentration (*16*), cell cycle time, autoradiographic techniques (*54*), messenger RNA synthesis (*55*), RNA polymerase activity (*17*), and DNA synthesis (*27*).

Several approaches commonly used with other *in vitro* test systems have been applied to limb bud cultures to activate the test compound. In an example of an *in vivo/in vitro* technique, the limb buds of embryos exposed *in vivo* to 6-mercaptopurine riboside (*20*) or to high levels of retinoic acid (*25*) developed characteristic abnormalities in organ culture, in an example of an *in vivo/in vitro* technique. Similarly, limb explants of mouse embryos were rendered sensitive to the action of thalidomide after pretreatment *in vivo* with specific mixed-function oxidase (MFO)-inducing agents (*27*). Neubert and Tapken (*56*) have already shown that certain enzyme systems like the aryl hydrocarbon hydroxylases can be induced at the organogenesis stage in mouse and rat embryos by MFO-inducing agents. The addition of an S-9 preparation from monkey liver (but not rat liver) with thalidomide and appropriate cofactors to rat limb buds in culture markedly reduced the growth of the limbs (*49*). In the case of stable, active metabolites such as those produced from cyclophosphamide, preincubation of the compound with liver microsomes and addition of the 100,000*g* supernatant or filtrate to the culture medium produced typical malformations of the limb during the cultivation (*8,15*). Addition of serum from cyclophosphamide-pretreated mice to the culture medium yielded similar results (*8*). Hamster embryo cells were not toxic when co-incubated with limb buds. The addition of cyclophosphamide to this system resulted in abnormal development of the cartilagenous skeleton (*44*).

Types of Compounds and Dose Response

The limb bud culture system has been tested with about 50 compounds, most of them biologically potent, representing several different chemical classes and biological activities, including known teratogens, carcinogens (e.g., alkylating agents), and those compounds with specific biochemical activities. Compounds with low water solubility such as thalidomide (*49*) and retinol (*57*) were dissolved in dimethyl sulfoxide (DMSO) and ethanol, respectively. For 5-fluorodeoxyuridine, the diluent was the culture medium itself (*45*).

Kwasigroch has indicated (*58*) that in his laboratory, the limb culture system has yielded few false negatives or false positives, although Barrach and Neubert (*8*) stated that false positive or false negative effects can be produced by modification or interference with components of the culture medium. Kochhar (*18*) reported that all the known teratogens tested altered the appearance of limb buds in a manner that could be termed "malformations." Kochhar (*59*) found that thalidomide dissolved in DMSO had a "strong inhibitory effect" on chondrogenesis of limb buds developing in culture. Although proline antagonists (e.g., azetidine and dehydroproline) also prevented normal chondrogenesis, such compounds have not been shown to be teratogenic in mice (*59*). Similarly, (bis[tri-*n*-butyltin]) oxide proved to be extremely potent in interfering with morphogenetic differentiation of the mouse limb in culture, although its embryotoxicity *in vivo* was low (*60*).

Neubert et al. (*46*) agreed with Kochhar (*18*) that specific malformations triggered *in vitro* should be distinguished from less specific effects such as failure of the explant to grow or form cartilage. For example, *in vitro* 6-mercaptopurine or the corresponding riboside produced syndactyly and phocomelia, while 5-bromodeoxyuridine (BudR) caused polydactyly of the mouse limb bud in a significant percentage of the explants. Other examples include 4-ketocyclophosphamide (distal limb reduction and hemimelic appearance), a stable metabolite of the teratogen cyclophosphamide (*53*), urethan (syndactyly) (*61*), and chlorambucil (severe long bone effects and less severe digital effects) (*62*).

Beta-aminopropionitrile (*18*) and acrolein (*63,64*) gave *in vitro* effects similar to those seen *in vivo* under highly specific conditions, e.g., at particular concentrations, or in the adult but not in the fetus. However, sometimes differences are subtle. After pregnant mice were injected with 6-diazo-5-oxo-L-norleucine (DON) or it was added to organ cultures on day-11 mouse limb buds, DON exerted its effects *in vivo* by interfering with purine metabolism but *in vitro* by interrupting glycosaminoglycan biosynthesis (*65*).

The age of the embryo used for the limb bud or the time after beginning of culture when the chemical was added affected the degree or type of abnormality. For example, urethan (*61*) did not cause digital malformations in day-11 limbs but induced syndactyly in day-12 forelimbs, and polydactyly in day-12 forelimbs and day-13 forelimbs and hindlimbs. Lesions caused by vitamin

A shifted from proximal to distal limb segments as the age of the donor limb bud increased (*57*). In another study (*25*), limbs from embryos of 29 to 32 somites were only mildly responsive to vitamin A, those from embryos of 33 to 38 somites incurred extensive teratogenic effects, while limbs from embryos with more than 40 somites escaped severe limb malformation except for digital effects. Also, limbs become increasingly resistant to the inhibitory effect of BudR (*48*) as they continue to differentiate.

Dose-response determinations were based upon (1) qualitative measurements, e.g., incidence of malformed limbs or of a specific abnormality, or loss of viability; (2) semiquantitative measurements based on a scoring system indicating specific or total regions deviating from the shape and size of normally developing limbs; and (3) quantitative relationships based on manual measurements of relative regional and total areas of the projected limb, or computerized measurements using image analysis systems to determine shapes and actual areas of specific bone and soft tissue areas. As already indicated, biochemical end points were also used. In most cases, a combination of two or more of these methods was applied to relate concentration of compound to response.

A typical example of a dose-response relationship using qualitative end points was reported for acrolein (*63*). At 1 μg/mL limb buds developed normally; at 10 μg/mL only a bending of the paw was seen; at 30 μg/mL only the metacarpalia, with all the phalanges missing, developed; and at 100 μg/mL differentiation and development of the paw skeleton were completely inhibited. With the acetoxy derivative of dimethylnitrosamine, a similar dose-response relationship was obtained, except that effective concentrations were seen in the 0.1–0.3 μg/mL range (*66*). Qualitative dose-response relationships were also seen for ribavirin (*14*). Kochhar (*18*) used cartilage area unit analysis to obtain dose-response relationships for BudR and ara-C; Neubert et al. (*47*) used the point system for scoring developmental abnormalities to compare dose responses for three purine antimetabolites.

The ability of the *in vitro* system to help decide which substances should be subjected to biological testing is corroborated by good correlations between the *in vitro* and *in vivo* effects of specific compounds and their less active derivatives or analogs. Using limb buds from embryos of pregnant rats treated with MFO-inducing agents, one derivative of thalidomide (EM-12) shown to be teratogenic after *in vivo* treatment produced malformations of mouse limbs in culture, but an apparently nonteratogenic thalidomide derivative (EM-87) failed to produce such effects (*27,67*). Cytosine arabinoside was a potent teratogenic agent *in vitro* but its inactive analog uridine arabinoside was not (*68*). All-*trans*-retinoic acid was considerably more effective in producing deformities than its less toxic analog, 13-cis-retinoic acid (*18*). Finally, of two antiglutamines, 6-diazo-5-oxonorleucine was more potent in producing growth inhibition and impaired chondrogenesis on cultured limb buds and teratogenesis *in vivo* than azaserine (*59*).

Reproducibility and Equipment and Personnel Requirements

Almost all users of the limb bud culture procedure agree on the high degree of reproducibility and precision obtained. To reduce variability, there is also strong agreement (*8,16,20,34*) on the need for careful standardization of the methodology, especially the use of cultures derived from embryos within narrow ranges of age (days and sometimes hours) and stages (somite number).

Neubert and Barrach (*15*) placed so much emphasis on the essentiality of using limb buds of the same developmental stage (for uniformity of results) that a particular experimental culture series included limb buds from embryos of a somite stage with a divergence of not more than one somite allowed. Their protocol further specified that at least 15 limb buds had to be cultured under identical conditions and that limb buds from one litter were pooled, using 50% of the randomized explants as controls. The authors claimed that their staging system and optional culture conditions gave better reproducibility than *in vivo* methods and that abnormalities could be produced in more than 90% of the limbs with the test compound.

When the Trowell (stationary) technique was used in Neubert and Barrach's laboratory, one person routinely performed two to three sets of experiments per week, preparing 150 to 200 limb buds for cultivation per day, providing for 30 to 40 Petri dishes containing four to five explants per dish. At least 100 limb buds (hind and fore) are evaluated for each experimental condition (*40*). Of the more than 10,000 control limb buds that had been examined (using a chemically defined medium) for state of morphogenetic differentiation reached, 80 to 90% had developed into well-differentiated embryonic limbs, and of the 10 to 15% incompletely developed buds, few resembled typical drug-induced malformations. These authors define reproducibility as all the limb buds reaching the same developmental stage in culture (obtained optimally by starting with a stage of 41–43 somites). The scoring system developed by Neubert and Barrach (*15*) was used.

Kochhar (*14*), using his culturing procedure and method of cartilage area quantitation, obtained reproducible data among similarly treated limbs. In that laboratory (Kochhar, Jefferson Medical College, PA, personal communication, 1984) the limb buds (separated into left and right for treated and control groups) from one, or occasionally two, litters of mice are used per experiment. Two limbs are incubated on one filter placed in a dish, and at least three dishes are used per compound level. A control set and three to four levels of compound are run per assay. One technician prepares and processes the cultures for analysis. Preparation of the cultures takes about one afternoon, and an entire experiment takes 7 to 8 days. Explants are then ex-

amined for regional and total areas by computer-controlled image analysis.

Kwasigroch et al. (*50,58*) also obtained excellent reproducibility and precision with his submerged culture system and computerized image analysis system. Although intra-experiment variances were small, no information was given on inter-experiment variability. All the limb buds from one litter of mice are pooled and placed into one culture bottle. Seven litters are used per treatment, so that in a typical experiment requiring four levels of treatment and one solvent control, the limb buds from 35 litters are needed. Seven limbs are randomly selected from each culture bottle for evaluation (i.e., 49 limbs per dose). Because development of the limb bud is apparently more rapid with the submerged culture system, the explants can be removed for processing after only 3 days of culturing, a procedure that eliminates the requirement for a medium change and minimizes the number of manipulations, personnel hours, and supply expense. The entire assay, beginning with preparation of the explants for culture, takes 11 to 12 days.

Other investigators (*69*) using the suspension or submerged culture technique and chemically defined medium found that a high degree of reproducibility was obtained by randomizing and distributing the limb buds from the embryos of two mice into three flasks. One culture served as the control and the other two as the experimental assays. In this case, however, the end point constituted only morphological evidence of limb abnormalities.

Neubert and Bluth (*70*) demonstrated that limb buds from 11- or 12-day-old mouse embryos could be stored successfully in medium under sterile conditions in a refrigerator at approximately 4°C for periods of several weeks.

Although many investigators generally considered the limb bud culture system to be highly reproducible, Shepard and Shiota (*49*) experienced considerable variability of the surface area and protein content of cultured limbs grown for 6 days, even when taken from the same litter. They speculate that the variability may be partly due to limb bud size differences between litters. Lessmollmann et al. (*20*) stated that the number of somites is somewhat variable even within the embryos of the same litter, emphasizing the importance of careful staging of the embryos before their limb buds are used for culture. Earlier, Neubert et al. (*46*) found variation in the degree of development when they examined the results of different experiments performed over several months. Neubert et al. (*16*) also found that results varied considerably with different strains of mice. NMRI mice, a randomly bred strain, responded in a more reproducible way and achieved better development of the hand skeleton than did strain DBA/2J, used by another laboratory.

The type of equipment needed for carrying out limb bud culture procedures is similar to that required for other culture systems. Kwasigroch et al. (*50*) use only a dissecting microscope with a Plexiglas or similar cover for aseptically preparing the limbs for culture; other laboratories prefer the use of a laminar flow hood. Other basic equipment includes an incubator, rotator device (for submerged culture systems), autoclave, camera with an appropriate coupling device to allow attachment to the microscope, and a system for projecting microscope-viewed explants. An image analyzer is recommended for rapid and accurate determination of areas and shapes of the developed limb bud and its components.

Mechanisms

A major advantage of the limb bud culture system is that it reportedly responds to a number of teratogenic compounds like the developing limb after *in vivo* treatment (*16,19*). Thus, information on the mechanism of developmental toxicants may be obtained by studying their early biochemical effects on stages of the limb developing in culture. Although scientists disagree about use of the limb bud system to screen for potential teratogens and fetotoxicants, most of them agree that it can be used to investigate teratogenic phenomena, especially those related to chondrogenesis and morphogenesis, and to answer a number of questions about the nature of teratogens. Flint (*71*), however, states that limb bud cultures measure the effects of compounds on growth rather than on the mechanisms controlling the pattern of limb cartilage.

To answer some of these questions, the pattern of changes was carefully compared in the *in vitro* growth and differentiation of the limb bud after exposure to a compound *in vitro* (*57,72*) or at the same stage *in utero* (*25,50*). Results indicated that vitamin A probably acts directly on the limb bud to produce characteristic malformations and that suppression of the formation of chondroitin sulfate may be partly responsible for the morphological alterations. Zimmermann and Tsambaos (*73*), using electron microscopy, confirmed the direct effect of retinoids on chondrogenesis, but Kochhar (*74*) recently provided evidence casting some doubt as to this effect being solely or fundamentally responsible for the teratogenic action of this class of compounds. *In vitro* studies demonstrated that cyclophosphamide (*53*) requires metabolic activation, while 5-fluorodeoxyuridine (*46*) and thiobendazole (*75*) act directly to impair chondrogenesis. Use of specific metabolic inhibitors may reveal basic factors associated with normal development. For example, the first and second day cultures were found to be the most susceptible to α-amanitine, a typical inhibitor of transcription, and to cycloheximide, a direct-acting inhibitor of protein synthesis (*8*). Also, the following caused cartilage abnormalities when added *in vitro* to limb cultures: 6-diazo-5-oxo-L-norleucine, an inhibitor of purine and glycosaminoglycan biosynthesis (*76*); L-azetidine-2-carboxylic acid, an inhibitor of collagen synthesis (*12*); and 4-methylumbelliferyl-β-L-xylopyranoside, an inhibitor of proteoglycan biosynthesis (*77*).

Measurement of macromolecular biosynthesis in spe-

cific embryonic cell types after administration of known teratogenic agents can also aid in elucidating mechanisms of action. Cytosine arabinoside at a dose that caused 90% polydactyly in limbs inhibited DNA synthesis in nearly all the mesenchymal cells but only up to 50% in ectodermal cells of mouse limb buds (*78*).

Similar methods may aid in distinguishing between the direct and indirect or more complex action of a compound in producing specific teratogenic effects. Acetoxymethyl-methylnitrosamine (DMN-OAc) triggers preferential left-sided paw defects in mice only after *in vivo* administration. A good correlation was found between the relative *in vitro* DNA alkylation rates with ^{14}C-DMN-OAc of separately pooled right and left limbs from fetuses of DMN-OAc-treated mice and the asymmetric teratogenic response to this compound (*79*). In the case of a noncompound-related condition, genetically determined polydactyly in mouse embryos was found to be associated with increased *in vitro* DNA synthesis and cAMP-phosphodiesterase within the region of the limb bud peculiar to the prospective polydactylous region (*80*).

Biochemical methods for investigating mechanisms of action may require measurement of particular species of a class of compounds. The teratogen 6-mercaptopurine riboside (6-MPr) did not affect cartilage formation in extent but interfered with morphogenetic differentiation. Measurement of RNA polymerase in the developing limb *in vitro* showed that 6-MPr had little effect on RNA polymerase I, while the activity of α-amanitine-sensitive RNA polymerase was greatly increased (*17*).

Indirect immunofluorescence staining techniques have been developed to follow the appearance of specific proteins found in connective tissue and muscle (*81*). Type I, II, III, and IV collagens, proteoglycans, and myosin appear at different times or stages in specific areas or tissues of the developing limb bud in submerged culture, very similar to what occurs *in vivo*. Indirect immunofluorescence will now be available for examining the specific and temporal biochemical effects of compounds on morphogenetic differentiation in the limb bud culture system. The immunofluorescence technique for the detection of type II collagen has already been applied to characterize mouse hereditary chondrodystrophies in limb organ culture (*82*).

Summary and Recommendation for Future Work

Based on the reported observations, the developing mouse limb bud in culture will undergo growth, cellular differentiation, and tissue organization processes similar to those occurring *in vivo* in a reproducible manner. The system offers many advantages as a model system for studying the effects of test compounds on these processes individually or collectively. Large numbers of limb buds from carefully staged embryos can be cultured in a relatively short period of time and grown in a chemically defined medium for almost full morphogenetic expression. Because of this ability, the assay has the potential as a biological test system for the elucidation of mechanisms responsible for the teratogenic activity of specific compounds or classes of compounds. Because of the exclusion of maternal, nutritional, hormonal, and other confounding factors such as tissue interactions, results would have to be used with caution in risk analysis. Furthermore, the system is apparently hypersensitive to the effects of some of the chemicals tested (*50,58,59,83*). A possible shortcoming is that several known teratogens act at early stages of organogenesis (i.e., day 9 of mouse development), stages at which the limb cannot be satisfactorily cultured (*15*). Possibly the limb bud culture system should only be used as part of a battery of two or more test systems in any *in vitro* screening scheme.

One difficulty in evaluating the usefulness of the test system is the number of variations of the test being used. Laboratories therefore will need to make detailed data available so that measures of intra- and inter-experiment-variations can be critically assessed. Only minimal statistical evaluation is currently available in most published reports.

The two general approaches (static and submerged system) should be standardized so that laboratories experienced in the technique can carry out a comprehensive validation and comparison of the procedures with the use of positive and negative control compounds.

A weakness in the assay system at present is the unavailability of a reliable, nontoxic metabolic activation system. More work is needed in this area; once progress is made, the system should be further validated with one or more of these activation systems by using both qualitative and quantitative morphological and possibly biochemical end points. Most important, more data on percentages of false positive and false negative responses to test chemicals must be compiled before the predictive value of the technique can be assessed.

Reports that pathological variants of the skeleton, e.g., syndactyly and polydactyly, produced *in vivo* by specific chemicals can be similarly produced by these same chemicals (or their metabolites) *in vitro* in limb bud culture are impressive, but the final evaluation cannot be made until there is further documentation and duplication.

REFERENCES

1. Karkinen-Jaaskelainen, M., and Saxen, L. Advantages of organ culture techniques in teratology. In: Tests of Teratogenicity in Vitro (J. D. Ebert and M. Marois, Eds.), North-Holland Publishing Co., Amsterdam, 1976, pp. 275–284.
2. Raynaud, A. Effects generaux de la cytosine-arabino furanoside sur l'embryon des reptiles. Crit. Rev. Acad. Sci. Paris 291: 253–255 (1980).
3. Birge, W. J., Black, J. A., Westerman, A. G., and Ramey, B. A. Fish and amphibian embryos—a model system for evaluating teratogenicity. Fundam. Appl. Toxicol. 3: 237–242 (1983).
4. Honda, A., Iwama, M., Umeda, T., and Mori, Y. The teratogenic mechanism of 6-amino-nicotinamide on limb formation of chick

embryos: abnormalities in the biosynthesis of glycosaminoglycans and proteoglycans in micromelia. J. Biochem. 91: 1959–1970 (1982).
5. Salzgeber, B. Effects of nitrogen mustard on the development of chick limb buds. Studies on the genesis of limb malformation. In: Tests of Teratogenicity in Vitro (J. D. Ebert and M. Marois, Eds.), North-Holland Publishing Co., Amsterdam, 1976, pp. 417–434.
6. Elmer, W. A., Smith, M. A., and Ede, D. A. Immunohisto-chemical localization of cyclic AMP during normal and abnormal chick and mouse limb development. Teratology 24: 215–223 (1981).
7. Merker, H. J. Significance of the limb bud culture system for investigations of teratogenic mechanisms. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1975, pp. 161–199.
8. Barrach, H. J., and Neubert, D. Significance of organ culture techniques for evaluation of prenatal toxicity. Arch. Toxicol. 45: 161–187 (1980).
9. Strangeways, T. S. P., and Fell, G. B. Experimental studies on the differentiation of embryonic tissues growing *in vivo* and *in vitro*. I. The development of the undifferentiated limb-bud (a) when subcutaneously grafted into the post-embryonic chick and (b) when cultivated *in vitro*. Proc. R. Soc. Ser. B99: 340–366 (1926).
10. Shepard, T. H., and Bass, G. L. Organ culture of limb buds from riboflavin-deficient and normal rat embryos in normal and riboflavin-deficient media. Teratology 3: 163–167 (1970).
11. Kochhar, D. M. Effects of azetidine-2-carboxylic acid, a proline analog, on chondrogenesis in cultured limb buds. In: Metabolic Pathways in Mammalian Embryos During Organogenesis and its Modification by Drugs (R. Bass, F. Beck, H. J. Merker, D. Neubert, and B. Randham, Eds.), Freie Universitat Press, Berlin, 1970, pp. 447–482.
12. Aydelotte, M. B. Influence of azetidine-2-carboxylic acid on developing mouse limb cartilage *in vitro*. Anat. Rev. 169: 269–270 (1971).
13. Aydelotte, M. B., and Kochhar, D. M. Development of mouse limb buds in organ culture: chondrogenesis in the presence of a proline analog, 1-azetidine-2-carboxylic acid. Dev. Biol. 28: 191–201 (1972).
14. Kochhar, D. M. Embryonic organs in culture. In: Teratogenesis and Reproductive Toxicology (E. M. Johnson and D. M. Kochhar, Eds.), Springer-Verlag, New York, 1983, pp. 301–314.
15. Neubert, D., and Barrach, H. J. Techniques applicable to study morphogenetic differentiation of limb buds in organ. In: Methods in Prenatal Toxicology (D. Neubert, H. J. Merker, and T. E. Kwasigroch, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1977, pp. 241–251.
16. Neubert, D., Merker, H. J., Barrach, H. J., and Lessmollmann, U. Biochemical and teratological aspects of mammalian limb bud development in vitro. In: Tests of Teratogenicity in Vitro (J. D. Ebert and M. Marois, Eds.), North-Holland Publishing Co., Amsterdam, 1976, pp. 335–365.
17. Barrach, H. J., Rautenberg, M., Tapken, S., and Neubert, D. Some biochemical characteristics of mouse limb buds differentiating in organ culture. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1975, pp. 114–132.
18. Kochhar, D. M. Embryonic limb bud organ culture in assessment of teratogenicity of environmental agents. Teratog. Carcinog. Mutagen. 2: 303–312 (1982).
19. Brown, N. A., and Fabro, S. E. The *in vitro* approach to teratogenicity testing. In: Development Toxicology (K. Snell, Ed.), Praeger Publishers, New York, 1982, pp. 31–57.
20. Lessmollmann, U., Neubert, D., and Merker, H. J. Mammalian limb buds differentiating in vitro as a test system for the evaluation of embryotoxic effects. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1975, pp. 99–113.
21. Merker, H. J., Kruger, U., and Zimmermann, B. Simulation of limb bud skeletogenesis in vitro. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 119–133.
22. Kochhar, D. M. Embryo explants and organ cultures in screening of chemicals for teratogenic effects. In: Developmental Toxicology (C. A. Kimmel and J. Buelke-Sam, Eds.), Raven Press, New York, 1981, pp. 303–319.
23. Kochhar, D. M. Elucidation of mechanisms underlying experimental mammalian teratogenesis through a combination of whole embryo, organ culture and cell culture methods. In: Test of Teratogenicity in Vitro (J. D. Ebert and M. Marois, Eds.), North-Holland Publishing Co., Amsterdam, 1976, pp. 485–494.
24. Agnish, N. D., and Kochhar, D. M. Direct exposure of postimplantation mouse embryos to 5-bromodeoxyuridine in vitro and its effect on subsequent chondrogenesis in the limbs. J. Embryol. Exp. Morphol. 36: 623–638 (1976).
25. Kochhar, D. M., and Aydelotte, M. B. Susceptible stages and abnormal morphogenesis in the developing mouse limb, analysed in organ culture after transplacental exposure to vitamin A (retinoic acid). J. Embryol. Exp. Morphol. 31: 721–734 (1974).
26. Fell, H. B. Experiments on skeletal growth and development in vitro in relation to the problem of phocomelia. Proc. R. Soc. London 118: 133–154 (1935).
27. Neubert, D., and Krowke, R. Effect of thalidomide derivatives on limb development in culture. In: Limb Development and Regeneration, Part A (J. F. Fallon and A. I. Caplan, Eds.), Alan R. Liss, New York, 1983, pp. 387–397.
28. Rajan, K. T. Human organ culture: applications in the screening of teratogenic drugs. Exp. Embryol. Teratol. 1: 65–89 (1974).
29. Lowther, D. A., Handley, C. J., and Gundalach, A. Effect of salicylic acid on articular cartilage in organ culture. Pharmacology 17: 50–55 (1978).
30. Saxen, I., and Saxen, L. Organ culture in teratology: closure of the palatal shelves as a model system. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1975, pp. 84–98.
31. Heggie, A. D. Growth inhibition of human embryonic and fetal rat bones in organ culture by Rubella virus. Teratology 15: 47–56 (1977).
32. Rapaka, R. S., Parr, R. W., Liu, T.-Z., and Bhatnagar, R. S. Biochemical basis of skeletal defects induced by hydralazine: Inhibition of collagen synthesis and secretion in embryonic chicken cartilage in vitro. Teratology 15: 185–194 (1977).
33. Levitt, D., Ho, P.-L., and Dorfman, A. Effect of 5-bromodeoxyuridine on ultrastructure of developing limb-bud cells in vitro. Dev. Biol. 43: 75–90 (1975).
34. Wilk, A. L., Greenberg, J. H., Horigan, E. A., Pratt, R. M., and Martin, G. R. Detection of teratogenic compounds using differentiating embryonic cells in culture. In Vitro 16: 269–276 (1980).
35. Ahrens, P. B., Solursh, M., and Reiter, R. S. Stage related capacity for limb chondrogenesis in cell culture. Dev. Biol. 60: 69–82 (1977).
36. Byrne, D. H., and Kitos, P. A. Teratogenic effects of cholinergic insecticides in chick embryos. IV. The role of tryptophan in protecting against limb deformities. Biochem. Pharmacol. 32: 2881–2890 (1983).
37. Hassell, J. R., and Horigan, E. A. Chondrogenesis: a model developmental system for measuring teratogenic potential of compounds. Teratog. Carcinog. Mutagen. 2: 325–331 (1982).
38. Herken, R. The influence of D-penicillamine on the proliferation rate of cells from the upper limb bud of mouse embryos in vitro. Teratology 15: 159–162 (1977).
39. Trowell, O. A. A modified technique for organ culture *in vitro*. Exp. Cell. Res. 6: 246–248 (1954).
40. Lesmollmann, U., Hinz, N., and Neubert, D. In vitro system for toxicological studies on the development of mammalian limb buds in a chemically defined medium. Arch. Toxicol. 36: 169–176 (1976).
41. Kwasigroch, T. E., and Skalko, R. G. Development of mouse limbs in organ culture: dose dependent retinoic acid-induced defects evaluated using image analysis. In: Limb Development and

Regeneration, Part A (J. F. Fallon and A. I. Caplan, Eds.), Alan R. Liss, New York, 1983, pp. 335–344.
42. Zimmermann, B., Neubert, D., Bachmann, D., and Merker, H. J. Induction of skeletal malformation in organ cultures of mouse limb buds. Experientia 31: 227–228 (1975).
43. Manson, J. M., and Simons, R. In vitro metabolism of cyclophosphamide in limb bud culture. Teratology 19: 149–158 (1979).
44. Pelkonen, O. The differentiation of drug metabolism in relation to developmental toxicology. In: Developmental Toxicology (K. Snell, Ed.), Praeger Publishers, New York, 1983, pp. 166–186.
45. Knudsen, T. B., and Kochhar, D. M. Limb development in mouse embryo. III. Cellular events underlying the determination of altered skeletal patterns following treatment with 5-fluoro-2-deoxyuridine. Teratology 23: 241–251 (1981).
46. Neubert, D., Tapken, S., and Merker, H. J. Induction of skeletal malformations in organ cultures of mammalian embryonic tissues. Naunyn-Schmiedebergs Arch. Pharmakol. 286: 271–282 (1974).
47. Neubert, D., Lessmollmann, U., Hinz, N., Dillman, I., and Fuchs, G. Interference of 6-mercaptopurine riboside, 6-methylmercaptopurine riboside and azathioprine with the morphogenetic differentiation of mouse extremities *in vivo* and in organ culture. Naunyn-Schmiedebergs Arch. Pharmakol. 298: 1093–1105 (1977).
48. Agnish, N. D., and Kochhar, D. M. Direct exposure of mouse embryonic limb buds to 5-bromodeoxyuridine in vitro and its effect on chondrogenesis: increasing resistance to the analog at successive stages of development. J. Embryol. Exp. Morphol. 36: 639–652 (1976).
49. Shepard, T. H., and Shiota, K. Bioactivation of thalidomide by a monkey liver fraction in a rat limb culture system. In: Limb Development and Regeneration, Part A (J. F. Fallon and A. I. Caplan, Eds.), Alan R. Liss, New York, 1983, pp. 377–385.
50. Kwasigroch, T. E., Skalko, R. G.,and Church, J. K. Mouse limb bud development in submerged culture: quantitative assessment of the effects of in vivo exposure to retinoic acid. Teratogen. Carcinogen. Mutagen. 4: 311–326 (1984).
51. Lilja, S., and Schimmelpfennig, K. Studies on glycosaminoglycans in cultured mouse limb buds. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, pp. 241–249 (1975).
52. Barrach, H. J., Mark, K. V. D., and Gay, S. Location of type I and type II collagen in developing limb buds of mammalia. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1975, pp. 145–148.
53. Manson, J. M., and Smith, C. C. Influence of cyclophosphamide and 4-ketocyclophosphamide on mouse limb development. Teratology 15: 291–300 (1977).
54. Herken, R. Autoradiographic investigations with ^{3}H-thymidine in limb bud cultures. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1975, pp. 200–212.
55. Rautenberg, P., and Neubert, D. Methods for the evaluation of mRNA fractions synthesized in limb buds differentiating in organ culture. In: New Approaches to the Evaluation of Abnormal Embryonic Development (D. Neubert and H. J. Merker, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1975, pp. 133–134.
56. Neubert, D., and Tapken, S. Some data on the induction of monooxygenases in fetal and neonatal mouse tissue. In: Role of Pharmacokinetics in Prenatal and Perinatal Toxicology (D. Neubert, H. J. Merker, H. Nau, and J. Langman, Eds.), Georg Thieme Publishers, Stuttgart, W. Germany, 1978, pp. 69–76.
57. Nakamura, H. Digital anomalies in the embryonic mouse limb cultured in the presence of excess vitamin A. Teratology 16: 195–199 (1977).
58. Kwasigroch, T. E., Skalko, R. G., and Church, J. K. Development of limb buds in organ culture: examination of hydroxyurea enhancement of bromodeoxyuridine toxicity using image analysis. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 237–253.
59. Kochhar, D. M. The use of in vitro procedures in teratology. Teratology 11: 273–287 (1975).
60. Krowke, R., Bluth, U., and Neubert, D. In vitro studies on the embryotoxic potential of (bis[tri-*n*-butyltin])oxide in a limb bud organ culture system. Arch. Toxicol. 58: 125–129 (1986).
61. Yasuda, Y. Digital anomalies of mouse limbs induced by treatment with urethan in vitro. Teratology 15: 89–96 (1977).
62. Sadler, T. W., and Kochhar, D. M. Teratogenic effects of chlorambucil on in vivo and in vitro organogenesis in mice. Teratology 12: 71–78 (1975).
63. Stahlmann, R., Bluth, U., and Neubert, D. Effects of some "indirectly" alkylating agents on differentiation of limb buds in organ culture. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 207–222.
64. Stahlmann, R., Bluth, U., and Neubert, D. Effects of the cyclophosphamide metabolite acrolein in mammalian limb bud cultures. Arch Toxicol. 57: 163–167 (1985).
65. Greene, R. M., and Kochhar, D. M. Limb development in mouse embryos: protection against teratogenic effects of 6-diazo-5-oxo-L-norleucine (DON) in vivo and in vitro. Embryol. Exp. Morphol. 33: 335–370 (1975).
66. Bochert, G., Platzek, T., and Wiessler, M. Comparison of effects on limb development in vivo and in vitro using methyl (acetoxymethyl) nitrosamine. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 223–235.
67. Neubert, D., and Bluth, U. Limb bud organ cultures from mouse embryos after apparent induction of monooxygenases in utero. Effects of cyclophosphamide, dimethylnitrosamine and some thalidomide derivatives. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 175–195.
68. Manson, J. M., Dourson, M. L., and Smith, C. C. Effects of cytosine arabinoside on in vivo and in vitro mouse limb development. In Vitro 13: 434–442 (1977).
69. Stahlmann, R., Bluth, U., Wiessler, M., and Neubert, D. Interference of acetoxy-alkyl-nitrosamines with limb bud differentiation in organ culture. Arch. Toxicol. 54: 109–129 (1983).
70. Neubert, D., and Bluth, W. Feasibility of storing embryonic tissues for subsequent use in organ culture. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 171–173.
71. Flint, O. P. An assessment of the available in vitro techniques for detecting teratogens. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 561–565.
72. Nakamura, H. Analysis of limb anomalies induced in vitro by vitamin A (retinol) in mice. Teratology 12: 61–70 (1975).
73. Zimmermann, B., and Tsambaos, D. Retinoids inhibit the differentiation of embryonic mouse mesenchymal cells in vitro. Arch. Dermatol. Res. 277: 98–104 (1985).
74. Kochhar, D. M. Skeletal morphogenesis: comparative effects of a mutant gene and a teratogen. Prog. Clin. Biol. Res. 171: 267–281 (1985).
75. Tsuchiya, T., and Tanaka, A. Effects of thiabendazole on the mouse limb-bud organ and cell in culture. Toxicol. Lett. 30: 19–26 (1986).
76. Aydelotte, M. B., and Kochhar, D. M. Influence of 6-diazo-5-oxo-L-norleucine (DON), a glutamine analogue, on cartilaginous differentiation in mouse limb buds in vitro. Differentiation 4: 73–80 (1975).
77. Merker, H. J., Gunther, T. H., and Kruger, U. Effect of 4-methylumbelliferyl-β-D-xylopyranoside on the morphology of embryonic cartilage in limb bud cultures. Teratology 18: 291–310 (1978).
78. Krowke, R., Berg, P., and Merker, H. J. Effects of cytosine arabinoside, 6-amino-nicotinamide, and 6-mercaptopurine riboside on ectoderm and mesoderm of mouse limb buds. Teratology 15: 137–148 (1976).
79. Bochert, G., Platzek, T., Blankenburg, G., Wiessler, M., and Neubert, D. Embryotoxicity induced by alkylating agents: Left-

sided preponderance of paw malformations induced by acetoxymethyl-methylnitrosamine in mice. Arch. Toxicol. 56: 139–150 (1985).

80. Knudsen, T. B., Elmer, W. A., and Kochhar, D. M. Elevated rate of DNA synthesis and its correlation to cAMP-phosphodiesterase activity during induction of polydactyly in mouse embryos heterozygous for *Hemimelia-extra toe* (Hm^x). Teratology 31: 155–166 (1985).
81. Barrach, H. J., Grundman, K., Hinz, N., and Felies, A. Comparison of the differentiation of muscle and connective tissue of mouse limb buds in culture and in vivo: a morphological study by indirect immuno-fluorescence. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 135–159.
82. Brown, K. S., Barrach, H. J., Cranley, R. E., Greene, R., Kimata, K., Kleinman, H. K., and Pennypacker, J. P. Biochemical characterization of mouse hereditary chondrodystrophies in organ culture. In: Culture Techniques—Applicability for Studies on Prenatal Differentiation and Toxicity (D. Neubert and H. J. Merker, Eds.), W. de Gruyter, Berlin, 1981, pp. 225–267.
83. Kwasigroch, T. E., and Skalko, R. G. The teratogenic interaction of hydroxyurea and 5-bromodeoxyuridine examined with the aid of limb culture and image analysis. Fundam. Appl. Toxicol. 5: 1161–1173 (1985).