EMBRYO CULTURE IN TERATOLOGICAL SURVEILLANCE
AND 'SERUM PROTEINS IN DEVELOPMENT

MASTER

Progress Report
for Period 1979 to 1980

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July 1980

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Prepared For

The United States Department of Energy
under Contract No. EVO3139
(formerly EY-76-S-02-3139)

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INTRODUCTION

The present report covers the period from July 1979 to July 1980. In addition to this report the reader is referred to 3 reprints of papers which appeared this last spring and a preprint of a chapter that has been accepted for publication. Three papers were presented at the meeting of the Teratology Society last June and a paper will be presented at the Second International Congress on Cell Biology this September. The abstracts are enclosed.

The reader should note that this progress report was taken verbatim (i.e. xeroxed directly) from the renewal application submitted July 1980. More precisely it was taken from the "Background" section because the work accomplished last year is contained in that section. The reader should not be confused by the reference citations as they refer to the "Literature Cited" section of that report.

I. Teratological Surveillance

In our first studies, the feasibility of using rat embryo cultures to test the teratogenic activity of serum was considered. Rats were injected with cadmium or cyclophosphamide and at various intervals they were sacrificed for their blood serum. Using head fold stages and a 48 hour culture period, survival, abnormality frequency and growth changed in a reproducible and predictable manner (21). That is to say, survival increased, abnormalities decreased and growth increased when the serum used as a culture medium was taken at progressively later times following injection. We also compared the response of embryos to serum from injected animals with cadmium or cyclophosphamide added directly to serum taken from uninjected (control) rats. This comparison provided an additional bonus because with both teratogenic substances the embryo responses were quite distinct. The details have recently

appeared (21) and will not be reviewed in depth for this proposal, nevertheless, these differences in response (direct versus through the animal) have provided encouragement and a rationale to use serum as the body fluid of choice in the estimation of teratogenic activity. These differences also provide an interesting challenge to the use of embryo cultures with "directly" added chemicals as a means to evaluate the teratogenicity of the chemical.

Clearly, the possibility of extending this approach to human serum testing could have considerable clinical impact. However, previously reported attempts to culture rat embryos on human serum were unsuccessful (16). A major limiting factor was found to be glucose and once this was realized we proceeded to demonstrate teratogenic activity in human serum from subjects receiving anticonvulsant drugs or undergoing cancer chemotherapy (22).

The details of our first human studies have now been published (22). Needless to say, these observations with human serum have led to a reorganization of our research priorities with most of our effort directed at the assessment of the possible application of the rat embryo culture test to human health and particularly to problems related to development abnormalities and reproductive difficulties. To be as clear and precise as possible--allow me to restate that our initial objective has been to assess and to demonstrate the possible applications of the rat embryo culture test to human health. It should be fully appreciated that in dealing with human subjects much experimentation depends on the availability of material. Thus, in outlining the lines of experimentation the reader must appreciate both our desire to demonstrate as many applications as possible as well as the "long term" nature of human experimentation which depends on availability of suitable material. For example, we are constantly on the look-out for subjects willing to quit smoking, who are reliable and who are willing to contribute regular bleedings

to our cigarette clearance study. The following is a brief outline of the studies in progress.

A. Basis for human serum variation. From the start of our human experimentation variation between subjects was apparent in the ability of their serum to support embryo growth and normal development. Thus, we reported an embryo protein range of 42.8 to 117.3. ug/embryo depending on the subject and an abnormality frequency of essentially 3 of 19 non-treated subjects (22). This variation was greater than we had found for rats and monkeys and was apparent even though glucose content was adjusted to the same level for all subjects.

Our present approach to serum variation has been to analyze serum from those subjects which cause problems for the cultured embryos. In this way we hope to find bases for the variations and at the same time develop our analytical capabilities to detect and to define potential human teratogens. The first subject selected, a 20 year old female, was unique in that her serum unlike some 85 other subjects tested to date was embryo lethal. As early as 12 hours after the start of culture, embryos showed retarded growth and development and the yolk-sac showed degenerative changes. By 36 hours of culture embryos were usually dead. The following case study is presented with the hope that the reader will appreciate the "in depth" analytical work that can be achieved with this approach.

A "life style" evaluation found that the subject consumed large amounts (10 cups per day) of tea. She was asked to stop drinking tea and also given a commercial vitamin supplement. Over a three month period, no improvement was observed. At the same time measurements of such standard parameters as glucose, protein, osmotic pressure, etc., showed that her serum did not differ

from controls. When as little as 5% by volume of the "lethal" serum was added to "control" human serum (serum that grows excellent embryos) the embryos were still severely inhibited or died. This latter observation suggested that a toxic substance was involved rather than a nutrient deficiency or some type of imbalance of ions, etc. Next, we found that the problem could not be dialyzed away (suggesting either a molecular weight exceeding 5000 or a small molecule bound to a large one) nor could it be absorbed out with activated charcoal (possibly excluding steroid type molecules). Molecular size filtration (Amicon) indicated that the lethal substance exceeded a molecular weight of 100,000 (or was bound to such a large molecule) and the fact that "it" was destroyed by trypsin suggested that a protein was involved. High resolution two dimensional polyacrylamide gel electrophoresis, however, failed to show any differences between lethal and control human serum. The early action of the lethal serum specifically on the yolk-sac suggested that "it" could be a specific-immunological type of reaction. This has been supported by the ability of yolk-sacs but not whole embryos to absorb out the lethal activity. Second, and even more striking, was the observation that the lethal activity was localized in the 30% saturation ammonium sulfate serum fraction. As expected, acrylamide gel analysis showed that the 30% fraction consisted almost exclusively of the immunoglobulins (three bands representing the two heavy and a single light chain). We have also tested her mother and three sisters who were found not to have embryo lethal serum. Recently, Dr. Tom Sadler of Cincinnati University Medical School tested the lethal serum on mouse embryos in culture and could not distinguish the lethal from control serum. In summary, it appears that the cause of the embryo lethality for this subject is due to an immunological type reaction, associated with the immuno-

globulin fraction and reactive against the yolk-sac of the rat embryo. The question, does this have any human reproductive significance cannot be answered because the subject has not been pregnant. A preliminary report of this work was recently presented (23).

B. Clearance times for occupational exposures and environmental pollutants (cigarettes). One possible application of the rat embryo culture test might involve the detection of potential teratogens in the work place or general environment. In addition, for women who must work or wish employment where a risk might exist, serum teratogenic clearance times could be determined prior to the initiation of a pregnancy. Our first studies with these objectives in mind have involved subjects who smoke cigarettes and were willing to quit "cold turkey". Studies with 10 subjects extending for up to 35 days of bleeding have now been completed. Four of these 10 gave increased embryo size (protein content) and reduced abnormality frequencies following their last cigarette. The first day without an embryo abnormality came on days 4 or 5; maximum embryo growth was achieved on days 9, 10, 16 and 18; and increases in embryo protein comparing last cigarette to day of maximum growth were 29%, 59%, 61% and 80%. Four subjects failed to show improvement but these four had problems unrelated to smoking which could provide an explanation. For example, one was a petro-chemical worker who was taking medication for a heart condition, another was a diabetic taking insulin, the third was a light pipe smoker and the last was on birth control medication. This left two subjects who again failed to show an improvement after their last cigarette but in these two cases no explanation was apparent. Finally, one of the original four who showed clear improvement returned to smoking and again reduced embryo growth and increased abnormalities were observed. Additional

subjects will be added to this study as the total number is rather small but we are encouraged by the results and feel that they could represent a realistic picture of human variability in response to toxicity as well as human reliability in response to experimentation. A preliminary report of this work has been recently presented (24).

C. Adding primate metabolism to the rat embryo culture test (Thalidomide).

Although Thalidomide may have been a unique teratogen, the insensitivity of rodents compared to the extreme sensitivity of primates has remained an important consideration for the development of teratological testing. The use of non-human primates has been restricted by the cost of maintaining sufficient numbers of pregnant animals for testing. An obvious approach would be to expose monkey (or even human volunteers) to a potential teratogen and simply use their serum as a culture medium. In this way short term studies could be run repeatedly on a group of monkeys without the need for a long pregnancy period. To test the feasibility of this approach a collaboration was established last year with Drs. A. G. Hendrickx and R. Parker of the Primate Laboratory of the University of California at Davis. Employing procedures used for human serum (immediate centrifugation and blood sugar adjustment) we found that rat embryos could be cultured on monkey serum and that the stage of monkey menstrual cycle did not affect the ability of the serum to support embryo growth and development. (I might add, embryo growth was extremely uniform for some 15 of 17 monkeys used in this study.) Next, monkeys were given bananas containing enough Thalidomide for either 100 or 300 mg/kg doses and bled at intervals up to 12 hours. These experiments have now been essentially duplicated. The effect of serum from the 100 mg/kg series was generally slight causing some growth reduction most apparent at 6 hours but no

abnormalities. In one study, the 2 hour serum group gave severe growth retardation and embryo abnormalities. The results in the 300 mg/kg study were consistent in that the 6 hour group were most severely affected with growth reductions and abnormalities such as open neural tubes. The six hour peak of activity was in general agreement with studies by Schumacher et al (25) on plasma levels of Thalidomide in the monkey. Next, for comparison rats were given 300 mg/kg Thalidomide dissolved in DMSO by stomach tube and bled at various intervals. A slight growth reduction was observed at 6 hours but embryos were normal. Both monkey and rat experiments will be repeated but these results were encouraging. Dr. W. Scott has offered to provide us with various Thalidomide metabolites which when added to control serum could provide an interesting comparison and could help to identify the active metabolite. A preliminary report of this work was recently presented (26).

D. Variation in metabolism and the design of drug therapy (anticonvulsants). In our first human studies (22) one group that gave consistently abnormal embryos were those subjects receiving anticonvulsant drugs. Individuals with epilepsy have a frequency of abnormal children which has been about double (12-14%) the rest of the population. Many questions concerning this problem remain unanswered. For example, is the increased abnormality frequency the result of the medication or epilepsy or both; are some drugs safer than others for pregnant women and is there individual variation associated with the safety question; and finally what is the best animal model to use in the study of these questions? To some extent, these questions may not appear relevant to the D.O.E. mission. Nevertheless, I feel that in studying epilepsy some important generality might be observed which could contribute to the broad application of the rat embryo testing procedure.

Furthermore, individuals with epilepsy are available and must receive frequent medical testing. To date, we have been able to contact several hospitals in the New England area which have agreed to participate in our study. Already it has been possible to test several individuals who have epilepsy but have never taken medication. Furthermore, we have been able to locate individuals taking single drugs; an important consideration as epileptics frequently use several different anticonvulsants. Finally, we have started to give rats anticonvulsants and have developed capabilities with a GLC which will allow comparisons to be made between drug levels and the response of embryos. Interesting and encouraging results have been obtained already concerning such questions as abnormality frequencies with serum from non-medicated epileptics, striking differences between drugs in teratogenicity and differences between serum from rats and man with the same drug at the same concentration. The results are too preliminary for detailed discussion at this time.

E. High risk pregnancies and fetal wastage (some unexpected findings).

As will be discussed in the Scientific Scope (or proposed studies) Section, we propose to eventually devote a major effort to the study of serum from women who have a history of miscarriages and/or children born with congenital defects. Assuming that the serum from such subjects will lead to difficulties for the cultured embryos, analysis of the serum and the identification of the cause may not only be of help to the individual but could also lead to the identification of unsuspected teratogens. Thus, we have been quite excited to make some relevant observations along these lines. First, as previously noted, 15 of 17 monkeys had serum which supported excellent growth and normal development of the cultured embryos. Embryos cultured on serum from the other two monkeys were a bit small and quite abnormal. These two monkeys, unknown to

the embryo culture lab, had extended histories of reproductive failure and were soon to be destroyed. All of the others (with one minor exception) had successfully given birth. In still another situation, we received serum from a woman with a long history of miscarriages and again the embryos were small and abnormal. We have been most encouraged by these results.

II. Mechanisms Regulating the Synthesis of Serum Proteins

The initial problem was to determine which cell type or types constituting the yolk-sac synthesized serum proteins. Methods were devised to manually separate the 4-day yolk-sac (which is actually the area opaca) into endoderm, mesoderm and ectoderm layers. The isolated layers were incubated in ^{14}C -valine while the intact yolk-sac was incubated in ^3H -valine. The incubation media from each layer was then coelectrophoresed on polyacrylamide gels with the intact yolk-sac medium. Only the endoderm synthesized serum proteins and all of the proteins (transferrin and the three embryo specific serum proteins: α -globulin-a, α -globulin-b and prealbumin) synthesized by the intact yolk-sac were also synthesized by endoderm. This was confirmed by using antibody prepared against chick embryo serum (13).

As an extension of this approach we next developed procedures for the culture of pure endodermal cells. Although these cells stopped dividing soon after being placed into culture, they continued to synthesize and secrete serum proteins for periods of up to a week. The relative synthesis of the serum proteins changed in a manner comparable to the developmental changes found for the intact yolk-sac. Thus, transferrin synthesis remained constant, albumin synthesis increased and the synthesis of the embryo specific proteins decreased. Returning to the question of environmental influences on serum protein synthesis, we have tested the response of these cells to the proteins ovalbumin, conalbumin and insulin and clearly they altered the relative synthesis of serum proteins

(14). Thus, to some extent the extracellular environment can act directly on endoderm cells to regulate the synthesis (or possibly breakdown and/or secretion as well) of serum proteins. In any case, the combination of a single cell type, a well defined group of products and an environmental modulator presents a situation amenable to analysis of regulating mechanisms.