

# The use of whole rat embryo cultures to identify and characterize causes of reproductive failure

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**ABSTRACT** The most important problem facing human teratology today is to identify the actual causes of this health problem. We have used cultures of whole rat embryos to address this problem using blood sera from individuals at risk as embryo culture media for this purpose. Through serum fractionations and nutrient supplementations to the serum we have studied drugs (dilantin, valproic acid), nutrient deficiencies (methionine) and an embryotoxic autoantibody to the protein laminin. In addition to identifying these factors it has been possible to address their mechanisms of action and to provide recommendations for treatment.

**KEY WORDS:** *embryo cultures and reproductive failure*

## Introduction

Poor reproductive outcomes such as fetal demise during pregnancy, morphological defects at term and functional deficits throughout life have continued to present serious problems of health. The late James Wilson (Wilson, 1973) as well as others estimated that the causes for as many as 70% to 80% of all birth defects were unknown while even higher estimates could be made for the unknown causes of fetal demise and functional deficits. Thus, it has appeared that before mechanisms of abnormal development can be addressed or appropriate treatments can be formulated the identification of actual causes must be achieved. Injecting pregnant rodents with so called "model" drugs and chemicals as well as the study of rodent strains containing mutated genes have not contributed to the solution of the problem of identifying previously unknown and unsuspected causes of human reproductive failure. Epidemiological surveillance also has failed to identify causes of human reproductive failures because of its insensitivity to detect relatively small numbers of individuals who may have high sensitivities to particular adverse conditions. Thus, most causes of human reproductive failures have been identified by the submission of "adverse reaction reports" by physicians who by chance noted a reaction and by chance took the time to report their observations.

We have attempted a unique approach to this problem making use of whole rat embryo cultures exactly as described and developed by D.A.T. New and his associates (New, 1966). In our laboratory, we have removed rat embryos at nine and one-half days of gestation with yolk sacs intact but Reichert's membranes removed and have selected a narrow range of embryos within the

head fold stage for experiments. Cultures routinely last 48 h. The media in general have consisted of 90% sera and 10% water (v/v) containing various supplements or chemicals of interest. Sera routinely have been immediately centrifuged and heat inactivated (Steele and New, 1974). Sources of sera have included human subjects or animals such as monkeys, cows and rats. Only sera from different rats have been pooled prior to use. At the end of the culture period, embryos routinely have been examined with a dissecting microscope for gross morphological defects, immediately photographed, then either fixed for future histological analyses or prepared for protein and DNA contents determinations to provide estimates of growth (Klein *et al.*, 1978).

Although the details of these procedures have been described repeatedly, certain features that have contributed to the success of this approach should be appreciated. First, the culture technique has permitted embryo exposures to be studied during the most rapid and most active stages of development, organogenesis. Although many organ systems develop during this period, we have tended to concentrate on neurulation and, particularly, neural tube closure as our primary endpoint. This appeared justified as blind trials with monkey sera indicated that this morphological endpoint was a superior predictor of the animal's reproductive history than, for example, estimates of embryo growth or size (Klein *et al.*, 1982). Second, it has been possible to use relatively high concentrations of sera (90%) for embryo cultures which have not been achieved, for example, with cell cultures. This we felt increased the sensitivity of the embryo cultures as it allowed the detection of substances present at low concentrations. Finally, the ability to select embryos within a narrow range of developmental stages for experiments was found to be both critical and essential to achieve a reasonable

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degree of reproducibility within and between experiments (Klein *et al.*, 1978). For this reason, we have attempted to select particular stages (early, mid or late head fold) for entire studies. For example, mid head fold stage embryos have been selected for all studies with human sera.

## Nutrition

### Epilepsy

Through a unique series of experiments it was found that whole rat embryo cultures could be used to detect nutritional deficiencies of potential relevance to human pregnancy outcomes. First, by bringing sera glucose levels to 300 mg % (3 mg/ml) it became possible to successfully culture rat embryos on human sera (Chatot *et al.*, 1980). Additionally, when sera from patients receiving cancer chemotherapy or anticonvulsant medications were used for culture, embryotoxicities (teratogenicities) were observed. This provided the basis to attempt a large scale trial with sera from epileptics in order to determine if the high reproductive risk (fetal loss and birth defects) experienced by these individuals was related to their medication, nutrition or some other factor associated with epilepsy. Additionally, some comparative insights into the relative embryotoxicity of various anticonvulsants might be achieved as this study was limited to sera from subjects receiving single drug therapy (monotherapy).

Sera samples from 128 epileptics were tested in embryo cultures and their levels of anticonvulsants were determined which included phenobarbital, phenytoin, valproic acid and carbamazepine (Chatot *et al.*, 1984). Although sera samples from the phenobarbital group had a lower frequency of abnormal embryos than from the other drug groups, a relationship between "parent" drug levels and sera embryotoxicities was not observed. Thus, in an attempt to find the basis for the embryotoxicities, samples were dialyzed and retested on whole embryo cultures. However, as dialysis would be expected to remove not only potential drugs and their metabolites but also low molecular weight nutrients from the sera, a supplement containing water soluble vitamins, essential amino acids and glucose was added to the dialyzed serum samples prior to retesting (Chatot *et al.*, 1984). Now, many of the previously embryotoxic human epileptic serum samples supported normal rat embryo development. However, an important control experiment precluded interpretation. Thus, when the same mixture of nutrients was added to sera that had not been previously dialyzed, many of the previously embryotoxic sera samples again supported normal embryo development (about 60%). By systematically eliminating components from the nutrient mixture it was possible to demonstrate in several cases that the most limiting nutrients for rat embryo development on these sera were folic acid or methionine. Furthermore, direct chemical analyses of the sera showed that these nutrients were indeed at low levels in comparison to control samples.

Embryo cultures also have been used to identify the potential proximal teratogens for phenytoin, the most widely used anticonvulsant (Clapper and Klein, 1986). When whole rat embryos were cultured on sera from monkeys gavaged with phenytoin, embryotoxicity was observed. As free phenytoin was not toxic, serum samples were fractionated and each fraction was tested for embryotoxicity when added to control serum. In this manner, the toxicity was found to be associated with a phenytoin-serum protein

complex of 80,000 daltons which appeared essential for the transport of the drug to the embryos.

### Cows

In an attempt to obtain large volumes of inexpensive serum for whole rat embryo culture as well as to identify potential environmental toxins (cows consume a lot of environment), attempts were made to culture embryos on cow (bovine) serum. In general, all serum samples taken from cows living in a variety of distant locations caused cultured embryos to be exencephalic (open neural tubes) and dorsiflexed with fusion (abnormal body curvature) (Coelho *et al.*, 1989). When the same mixture of water soluble vitamins and essential amino acids that were used in the epilepsy study were now added to cow sera, embryo development was normal. Again, nutrients were eliminated sequentially from the supplement and this led to the identification of the amino acid methionine as the sole supplemental nutrient required to support normal rat embryo development on cow serum. Free methionine could not be detected in many samples of cow serum and the serum proteins themselves contained less methionine than was found in rat serum proteins.

That this response to methionine was related more to the methyl donor activity of this amino acid than to its role as a constituent of proteins, was suggested by the observation that some partial replacement of methionine could be achieved with choline chloride (a methyl donor) and that the requirement for methionine was limited to the first 18 h of culture; the period just preceding neural tube closure.

Furthermore, several observations implicated post-translational protein methylation as a key factor rather than DNA or RNA methylation (Coelho and Klein, 1990). For example, when embryos were cultured on diluted cow serum so that net increases in embryo protein content were not observed, neural tube closure could still be achieved if methionine was added to the diluted medium. Next, differences in two dimensional polyacrylamide gels were not apparent when neural tube proteins from plus and minus methionine cultured embryos were compared. Finally, actual reductions in the levels of neural tube protein methylated amino acids were detected in embryos cultured without methionine compared to those embryos given methionine.

### Drugs

Valproic acid, an anticonvulsant, has been recognized as a human teratogen that caused a variety of birth defects and particularly neural tube defects such as spina bifida. When rats were given methionine in their drinking water prior to as well as throughout their pregnancies and injected twice a day with valproic acid (650 mg/kg/day) on days 7, 8 and 9 of gestation resorption at 18 days of gestation were fewer (42%) than for those not given methionine (66%) (Nosel and Klein, 1992). However, unlike these *in vivo* studies the simple addition of methionine to the *in vitro* culture medium containing valproic acid (0.15 mg/ml) did not reduce the frequency of neural tube defects which remained at approximately 78% at the level of valproic acid used. Considering the possibility that the critical difference between *in vivo* and *in vitro* conditions involved exposures to elevated methionine at an earlier developmental stage for the *in vivo* group than for the *in vitro* group, embryos were removed for culture from pregnant rats that had been given methionine continuously in their drinking water starting

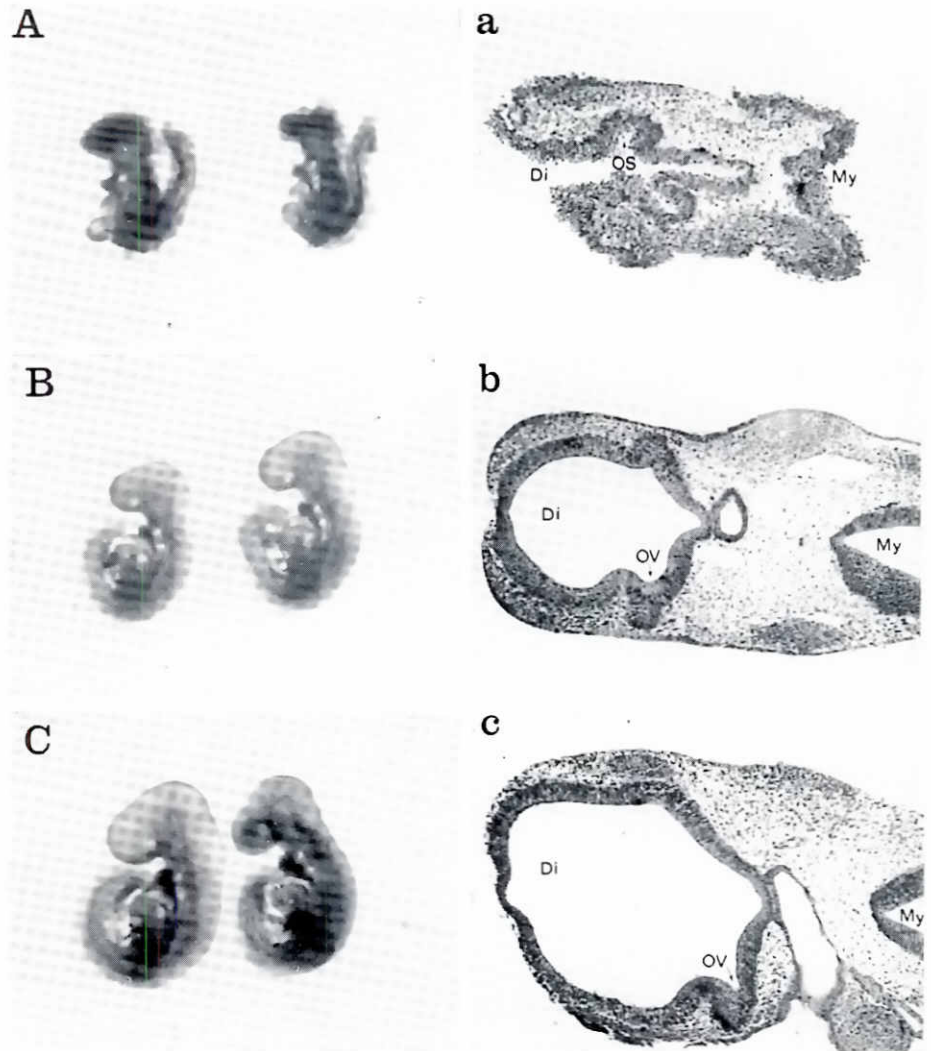
at least two weeks prior to breeding. The results were striking as these embryos exposed *in utero* to methionine exhibited neural tube defects of only 25% compared to 78% (as previously noted) of the embryos taken from untreated pregnant rats. These observations suggested the possibility that methionine exposure triggered molecular events precociously rendering the embryos less sensitive to the teratogenicity of valproic acid. This value of methionine was recently confirmed when reductions in spina bifida were found using a mouse model that developed such defects in response to valproic acid (Ehlers *et al.*, 1996). In contrast, it should be noted that folic acid failed to reduce the frequency of valproic acid induced neural tube defects in the mouse (Hansen *et al.*, 1995).

### Genes

Although not directly involved with whole embryo culture, studies with the Axd (axial defect) mouse mutant have been particularly relevant to the importance of methionine during development (Essien and Wannberg, 1993). When Axd/+ mice were injected with methionine (180 mg/kg or 70 mg/kg) on days 8 and 9 of gestation neural tube defects were reduced from 30% (uninjected) to 16%. Neither injections of folinic acid or B-12 reduced the incidences of neural tube defects in these mice. However, all mouse mutant strains have not responded so favorably to methionine as neural tube defects were not reduced in the Curly Tail strain (Van Straaten *et al.*, 1995).

### Mechanisms

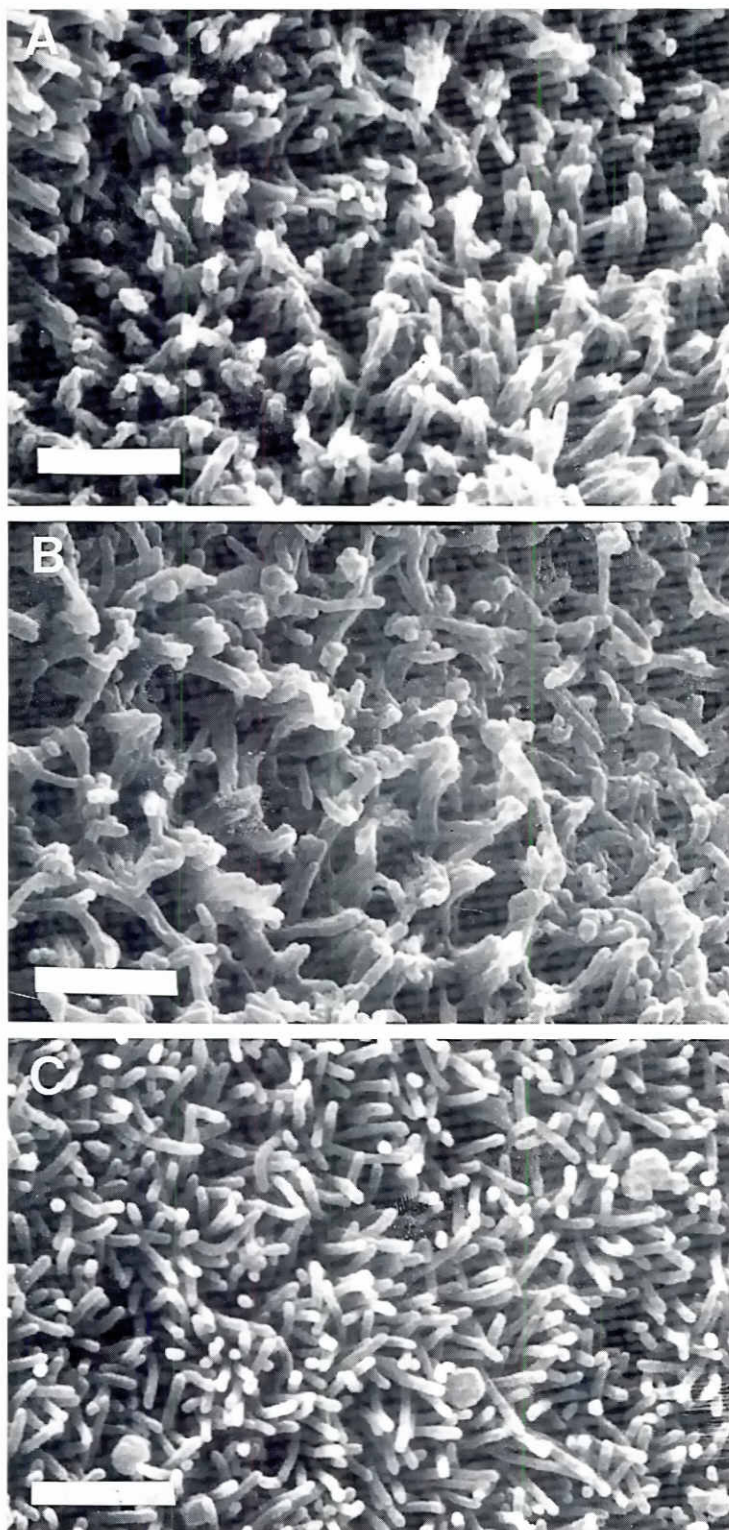
The ability to culture whole rat embryos on a medium lacking methionine (cow serum) has permitted the analyses of the role of this amino acid in neural tube closure. As previously noted in this review several lines of evidence implicated methionine in the post-translational methylation of neural tube proteins as a possible factor. Thus, to further substantiate this hypothesis embryos were cultured for 40 h on cow serum, transferred for 1 h to Tyrode's salts containing puromycin (reducing incorporation of S<sup>35</sup> methionine into proteins by 70%) and then <sup>14</sup>C-methyl labeled methionine was added in the continued presence of puromycin for an additional five hours of incubation (Moephuli *et al.*, 1997). Two dimensional polyacrylamide gels were then run and the dried gels were exposed to X-ray film. Three main spots were observed and based on their molecular weights and known methylations they were predicted to be actin, alpha-beta subunits of tubulin and the low molecular weight neurofilament protein. Western blots with antibodies to these proteins supported this prediction and analyses of protein hydrolysates by thin layer



**Fig. 1.** Rat embryos after 48 h of culture on serum samples from a single subject. Photographs on the left (A,B,C) (original magnification  $\times 12$ ) were taken of live embryos immediately after culture. Photographs on the right (a,b,c) (original magnification  $\times 100$ ) were taken of sections of embryos after Carnoy's fixation, paraffin sections (7  $\mu$ m), and staining with Ehrlich's hematoxylin and periodic acid-Schiff reagent. (A,a) Embryos cultured on serum without vitamin or amino acid supplements to either medium or diet. They had open neural tubes, poorly developed optic vesicles, and were dorsiflexed. (B,b) Embryos cultured on serum with nutrient supplement to serum (methionine, 25  $\mu$ g) but not to diet. These embryos were normal and had completed neural tube closure. (C,c) Embryos cultured on serum after dietary supplement of subject (500 mg/day, L-methionine) but without additional serum supplements. Embryos were normal with completed neural tube closure. Di, diencephalon; My, myelencephalon; OS, optic sulcus; OV, optic vesicle.

chromatography confirmed that embryo proteins contained radioactive methylated amino acids (3-methylhistidine, monomethyllysine, trimethyllysine and dimethylarginine).

It was now possible to study the direct effects of methionine deficiency on the distribution of these cytoskeletal proteins by indirect immunofluorescence. As has been previously reported for actin (Sadler *et al.*, 1982), this protein was first seen on the apical surfaces of the neuroepithelium cells and before neural tube closure fluorescence also became apparent in the basal regions of these cells as well as at the tips of the neural folds. In the absence of supplemental methionine the neural folds failed to turn in and fuse and although fluorescence was apparent still in the cytoplasm



**Fig. 2.** Scanning electron micrograph of the surface of the yolk sacs from embryos cultured for 24 h on sera from laminin immunized monkeys. **(A)** Serum from methionine responding monkey. **(B)** Serum from methionine non-responding monkey. **(C)** Serum from control monkey (BSA immunized). Note clumping of microvilli and reduced numbers of microvilli in **A** and **B**. (Bar, 1  $\mu$ m).

of the apical side of the neuroepithelial cells, fluorescence was neither apparent in the basal regions of the neuroepithelium cells nor at the tips of the folds. Tubulin responded similarly to actin and as yet it has not been possible to observe the neurofilament protein. Preliminary observations have indicated that the human teratogen, valproic acid also may limit this shift in actin and tubulin fluorescence. Although these studies would suggest that the methylation of these proteins and their distribution in the neuroepithelium cells was important for closure of the neural tube, further studies should show that these observations did not represent secondary responses to the adverse condition of methionine deficiency and that proteins less abundant than the three studied here were not involved in neural tube closure.

### Fetal loss

#### Monkeys

The species specificity of teratogens such as phenytoin and thalidomide suggested that it would be useful to culture rat embryos on monkey sera. For example, monkeys could be given a drug in question and their sera could be tested for teratogenicity using whole rat embryo cultures. This would permit the incorporation of primate metabolism into a test without the need for the animal to be pregnant and the animal could be reused once the clearance of the previous drug had been achieved. When sera from 18 female monkeys housed at the Primate Center of the University of California, Davis were tested by embryo culture, 16 supported normal rat embryo development while two caused embryos to be microcephalic, exencephalic (open neural tubes) and anophthalmic (Klein *et al.*, 1982). It was later learned that these two, unlike the other 16, had not completed pregnancies during two years of breeding. This study was extended to monkeys at the Primate Center of the University of Washington, Seattle and in further blind trials it was possible to distinguish sera from monkeys with excellent reproductive histories from sera of monkeys that had experienced considerable fetal loss (the rat embryo responses coincided for 8 of 12 good reproducers and 12 of 14 poor reproducers). As our interests now shifted we have not returned to our original objective of using monkeys with rat embryo cultures for drug testing.

Attempts were next made to identify sera factors in these monkeys that might be responsible for the fetal loss (Carey and Klein, 1989). Sera samples were first dialyzed and supplemented with essential amino acids and water soluble vitamins and although several serum samples responded favorably to this treatment, those samples remaining embryotoxic after this treatment received our interest and attention. Thus, the sera were separated into protein fractions by classical ammonium sulfate salting out techniques and each fraction was tested by addition to control sera. The immunoglobulin (IgG) fraction was implicated clearly as the toxic constituent. Indirect immunofluorescence showed that antibodies from the IgG fraction bound to Reichert's membrane and Western blots with solubilized Reichert's membranes implicated antibodies to the large extracellular matrix protein, laminin, as the toxic antibodies. (It should be noted that although Reichert's membrane is removed prior to embryo culture, it was used here for convenience as it contains a limited number of extracellular matrix

proteins. Yolk-sac endodermal cells have been known to synthesize laminin and laminin antibodies bind to the surfaces of these cells). We have assumed that the presence of these antibodies resulted from an autoimmune response. When monkeys with excellent reproductive histories were immunized with purified laminin, their sera became toxic to cultured rat embryos and they subsequently failed to reproduce (Weeks *et al.*, 1989).

### Women

Rat embryos were cultured on sera from 102 women who had histories of spontaneous abortions or normal pregnancy outcomes (Ferrari *et al.*, 1994). The frequencies at which subjects sera failed to support normal embryo development ranged from a low of 27% for those who had not experienced spontaneous abortions to a high of 89% for those who had experienced five or more fetal losses. When 48 of these teratogenic serum samples were retested in embryo cultures with supplements of water soluble vitamins and essential amino acid, 40 (83%) now supported normal embryo development. Now, 10 of these subjects received recommendation to take dietary supplements based on the whole rat embryo analyses of their sera. Sera from six of these 10 were found to support normal rat embryo development following their dietary supplements and all six subsequently completed normal pregnancies (Fig. 1). Over the past several years approximately 40 additional individuals completed pregnancies while taking L-methionine (500 mg/day, half with breakfast and the other half with dinner) but it has been estimated that 866 recurrent aborters (half taking methionine and half placebo controls) would be required for a statistically sound study. It should be noted that in similar studies with rather small numbers of individuals, one group found a comparable difference between the ability of sera from aborters and non-aborters to support rat embryo development (Abir *et al.*, 1994) while a second group failed to see this difference (Scialli *et al.*, 1993). However, this latter group found that sera from individuals changed from embryotoxic to non-toxic with dietary supplements.

### The induction of autoantibodies to laminin

Although laminin antibodies were found to be one embryotoxic factor in the sera of monkeys with poor reproductive histories, it was only possible to assume that the antibodies were the result of an autoimmune reaction. Autoantibodies, given the appropriate genotype, have been considered to result from conditions that compromised the ability of the immune system to distinguish antigens from self and non-self. Two approaches have been used to study this condition. First, individuals who have Chagas' disease (a protozoan infection) have been found to have circulating antibodies that cross react with laminin. Sera from individuals with this disease were found to be toxic to cultured rat embryos and that this toxicity could be reduced by prior absorption of the sera with purified laminin (Robbins *et al.*, 1991). Furthermore, when affinity purified anti-laminin antibodies were added to control sera for embryo culture they were embryotoxic. In the second approach Brown Norway rats were used as they were known to be sensitive to mercury induced glomerulonephritis and as a result produced antibodies against extracellular matrix proteins such as laminin. Sera from these mercury treated Brown Norway rats were toxic to cultured rat embryo and, again, affinity purified laminin antibodies when added to control sera were embryotoxic (Chambers and Klein, 1993).

These experiments with mercury and the Brown Norway rat have implicated a potential etiology for fetal loss and birth defects that should be fully appreciated. Thus, exposures to a variety of substances including environmental pollutants, infectious agents and drugs have been known to induce autoimmune diseases. If this occurred in a young child, such autoantibodies could persist throughout the individual's life at sub-clinical levels. However, during her childbearing years the levels could well be sufficient to cause fetal loss, structural defects at birth or functional deficits throughout the life of her children. In studies that have been described with monkeys, there were no clinical manifestations that antilaminin antibodies were present (both laminin immunized and "natural occurring" laminin antibodies) except for continuous fetal loss.

### Laminin epitopes

In studies with sera from rats immunized with laminin (Weeks and Klein, 1989) as well as studies with humans the presence of laminin antibodies alone was not sufficient to predict the toxicity of the sera to cultured rat embryos. As antibody avidities and levels (as measured by ELISA) were also of no predictive value for embryotoxicity, differences in epitopes (or determinant) recognized by the antibody were considered. In the first approach, six different monoclonal antibodies to laminin were added to control serum for rat embryo cultures (Rasmussen *et al.*, 1994). Four were found to be embryotoxic (teratogenic or lethal) and two at comparable levels were non-toxic. This suggested differences in antibody epitope specificity could be involved in the differences in antilaminin antibody embryotoxicity. To further substantiate this point, monkeys with excellent reproductive histories were immunized with the synthesized laminin amino acid sequences YIGSR, RGD and IKVAV (Chambers *et al.*, 1995a). Serum from the monkey immunized with YIGSR became toxic to cultured rat embryos and this monkey subsequently experienced fetal loss. RGD antibody sera again caused embryotoxicity in cultures but this monkey became infertile. Finally, IKVAV antibody sera not only allowed normal rat embryo development in cultures but also allowed for completion of pregnancy with a normal outcome. These studies demonstrated the importance of epitope specificity in the toxicity of antibodies and the importance of epitope mapping in predicting the reproductive risk of a particular antibody.

### Mechanism of antilaminin antibody embryotoxicity and interaction with methionine

Sera from two monkeys immunized with laminin were found to be highly toxic to cultured rat embryos (Chambers *et al.*, 1995b). This toxicity could be completely overcome for one of the monkeys by either adding methionine to her sera for culture or by placing the monkey on a methionine supplemented diet. These treatments did not alter the serum toxicities of the second monkey. When viewed by electron microscopy, antibody binding to the surfaces of the yolk-sac endodermal cells was more dense and continuous along the surfaces of the cells for the non-responder to methionine than to the responder. In addition, the yolk-sac cell surface microvilli from the non-responder were more clumped and reduced in surface area than the responder (Fig. 2). Studies then confirmed that the uptake of methionine (as well as other nutrients) was reduced to a greater extent by antibodies from the non-responder than from the responder. If such antibodies also interfered with the passage of nutrients across the placenta, it might be useful to

provide methionine supplements to individuals with autoimmune problems (such as systemic lupus erythematosus) who have experienced fetal loss. It should be noted that studies have been initiated to define the laminin cell surface receptors involved in the toxicity of these antibodies (Jeong *et al.*, 1995).

It also should be noted that it has been possible to isolate IgG fractions from human plasmapheresis plasma and to add precise amounts (4, 7.5 and 11 mg/ml) to control rat sera for testing in whole rat embryo cultures (Nadler *et al.*, 1995). In one study, 4 of 6 IgG fractions isolated from the plasma of individuals with systemic lupus erythematosus were embryotoxic while only one of six fractions from non-lupus individuals was embryotoxic. This study should provide a basis for attempts to identify more precisely specific embryotoxic antibodies in an attempt to identify the actual risk factor associated with lupus fetal loss.

### Methionine and folic acid

Following the outstanding work of Smithells with individuals on poor diets (Smithells, *et al.*, 1980), at least two major epidemiological studies showed that dietary supplements of folic acid could reduce the occurrence and recurrence of neural tube defects. On the other hand, whole rat embryo cultures as described here have directed attention to the amino acid, methionine, in neural tube defects. This interplay between epidemiology and whole rat embryo cultures has been of some interest not only because of the striking differences in approaches used but also of considerable significance in regard to selecting the most appropriate treatment. The epidemiologists have suggested that folic acid acts to reduce levels of circulating homocysteine, a substance that has been implicated in problems of vascular disease. However, in whole rat embryo cultures homocysteine was not found to be toxic at elevated circulating levels (Van Aerts *et al.*, 1994). Furthermore, when folic acid has acted to reduce homocysteine it turns homocysteine into methionine. In order to provide methionine for methylation reactions, one should consider ingesting foods high in methionine such as chicken eggs or other methyl donors while folate needed to reduce homocysteine should direct one to consume leafy vegetables. How these differences ultimately "play out" should be of interest and of some significance.

### Conclusions

The objective of this review has been to place the technique of whole rat embryo cultures in the broadest perspective of identifying actual reproductive risk factors, providing insights into their mechanisms of developmental toxicity and, ultimately, suggesting logical directions for treatment. Studies with two previously unknown and unsuspected factors have been described here (methionine and antilaminin antibodies). There must be many more. Hopefully, others may turn to whole rat embryo cultures in the manner that has been described in this brief review.

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### References

- ABIR, R., ORNOY, A., BEN HUR, H., JAFFE, P. and PINUS, H. (1994). The effects of sera from women with spontaneous abortions on the *in vitro* development of early somite stage rat embryos. *Am. J. Reprod. Immunol.* 32: 73-81.
- CAREY, S.W. and KLEIN, N.W. (1989). Autoantibodies to laminin and other basement membrane proteins in sera from monkeys with histories of reproductive failure identified by cultures of whole rat embryos. *Fertil. Steril.* 51: 711-718.
- CHAMBERS, B.J. and KLEIN, N.W. (1993). Role of laminin autoantibodies on the embryo toxicity of sera from mercuric chloride treated Brown Norway rats. *Reprod. Toxicol.* 7: 333-341.
- CHAMBERS, B.J., KLEIN, N.W., CONRAD, S.H., RUPPENTHAL, G.C., SACKETT, G.P., WEEKS, B.S. and KLEINMAN, H.K. (1995a). Reproduction and sera embryotoxicity after immunization of monkeys with the laminin peptides YIGSR, RGD, and IKVAV. *Proc. Natl. Acad. Sci. USA* 92: 6818-6822.
- CHAMBERS, B.J., KLEIN, N.W., NOSEL, P.G., KHAIRALLAH, L. and ROMANOW, J. (1995b). Methionine overcomes neural tube defects caused by antilaminin antibodies. *J. Nutr.* 125: 1587-1599.
- CHATOT, C.L., KLEIN, N.W., CLAPPER, M.L., RESOR, S.R., SINGER, W.D., RUSSMAN, B.S., HOLMES, G.L., MATTSON, R.H. and CRAMER, J.A. (1984). Human serum teratogenicity studied by rat embryo culture: epilepsy, anticonvulsant drugs and nutrition. *Epilepsia* 25: 205-216.
- CHATOT, C.L., KLEIN, N.W., PIATEK, J. and PIERRO, L.J. (1980). Successful culture of rat embryos on human serum: use in the detection of teratogens. *Science* 207: 1471-1473.
- CLAPPER, M.L. and KLEIN, N.W. (1986). Identification of a teratogenic drug protein complex in sera of phenytoin-treated monkeys. *Epilepsia* 27: 685-696.
- COELHO, C.D., WEBER, J.A., KLEIN, N.W., DANIELS, W.G. and HOAGLAND, T.A. (1989). Whole rat embryos require methionine for neural tube closure when cultured on cow serum. *J. Nutr.* 119: 1716-1725.
- COELHO, C.N.D. and KLEIN, N.W. (1990). Methionine and neural tube closure in cultured rat embryos: morphological and biochemical analyses. *Teratology* 42: 437-451.
- EHLERS, K., ELMAZAR, M.M.A. and NAU, H. (1996). Methionine reduces the valproic acid-induced spina bifida rate in mice without altering valproic acid kinetics. *J. Nutr.* 126: 67-75.
- ESSIEN, F.B. and WANNBERG, S.L. (1993). Methionine but not folic acid or vitamin B-12 alters the frequency of neural tube defects in *Axd* mutant mice. *J. Nutr.* 123: 27-34.
- FERRARI, D.A., GILLES, P.A., KLEIN, N.W., NADLER, D., WEEKS, B.S., LAMMI-KEEFE, C.J., HILLMAN, R.E., CAREY, S.W., YING, Y-K., MAIER, D., OLSEN, P., WEMPLE, D.W., GREENSTEIN, R., MUECHLER, E.K., MILLER, R.K. and MARIONA, F.G. (1994). Rat embryo development on human sera is related to numbers of previous spontaneous abortions and nutritional factors. *Am. J. Obstet. Gynecol.* 170: 228-236.
- HANSEN, D.K., GRAFTON, T.F., DIAL, S.L., GEHRING, T.A. and SIITONEN, P.H. (1995). Effect of supplemental folic acid on valproic acid-induced embryotoxicity and tissue zinc levels *in vivo*. *Teratology* 52(5): 277-285.
- JEONG, B.S., KLEIN, N.W. and BALDWIN, M.T. (1995). The interference of laminin-receptor interaction by laminin antibodies. *Mol. Biol. Cell* 6 S: 166a.
- KLEIN, N.W., PLENEFISCH, J.D., CAREY, S.W., FREDRICKSON, W.T., SACKETT, G.P., BURBACHER, T.M. and PARKER, R.M. (1982). Serum from monkeys with histories of fetal wastage cause abnormalities in cultures of whole rat embryos. *Science* 215: 66-69.
- KLEIN, N.W., VOGLER, M.A., CHATOT, C.L. and PIERRO, L.J. (1978). The use of cultured rat embryos to evaluate the teratogenic activity of serum: cadmium and cyclophosphamide. *Teratology* 21: 199-208.
- MOEPHULI, S.R., KLEIN, N.W., BALDWIN, M.T. and KRIDER, H.M. (1997). Effects of methionine on the cytoplasmic distribution of actin and tubulin during neural tube closure in rat embryos. *Proc. Natl. Acad. Sci. USA* 94: 547-548.

- NADLER, D.M., KLEIN, N.W., ARAMLI, L.A., CHAMBERS, B.J., MAYES, M. and WENER, M.H. (1995). The direct embryotoxicity of immunoglobulin G fractions from patients with systemic Lupus erythematosus. *Am. J. Reprod. Immunol.* 34: 349-355.
- NEW, D.A.T. (1966). Development of rat embryos cultured in blood sera. *J. Reprod. Fertil.* 12: 509-524.
- NOSEL, P.G. and KLEIN, N.W. (1992). Methionine decreases the embryotoxicity of sodium valproate in the rat: *in vivo* and *in vitro* observations. *Teratology* 46: 499-507.
- RASMUSSEN, M.V., KLEIN, N.W., ABRAHAMSON, D.R. and CHUNG, A.E. (1994). Effects of laminin monoclonal antibodies on the development of cultured rat embryos. *Teratology* 49: 20-28.
- ROBBINS, B., KLEIN, N.W. and CAVALCANTI, H. (1991). Toxicity of sera from individuals with Chagas' disease to cultured rat embryos: role of antibodies to laminin. *Teratology* 44: 561-570.
- SADLER, T.W., GREENBERG, D., COUGHLIN, P. and LESSARD, J.L. (1982). Actin distribution patterns in the mouse neural tube during neurulation. *Science* 215: 172-174.
- SCIALLI, A.R., FLYNN, T.J. and GIBSON, R.R. (1993). Rat embryo culture to detect nutritional deficiency in women with poor reproductive histories. *Reprod. Toxicol.* 7: 581-587.
- SMITHELLS, R.W., SHEPPARD, S., SCHORAH, C.J., SELLER, M.J., NEVIN, N.C., HARRIS, R., READ, A.P. and FIELDING, D.W. (1980). Possible prevention of neural tube defects by periconceptual vitamin supplementation. *Lancet* 1: 339-340.
- STEELE, C.E. and NEW D.A.T. (1974). Serum variants causing formation of double hearts and other abnormalities in explanted rat embryos. *J. Embryol. Exp. Morphol.* 31: 707-719.
- VAN AERTS, L.A.G.J.M., BLOM, H.J., DEABREU, R.A., TRIJBELS, F.J.M., ESKES, T.K.A.B., PEEREBOOM-STEGEMAN, J.H.J.C. and NOORDHOEK, J. (1994). Prevention of neural tube defects by and toxicity of L-homocysteine in cultured postimplantation rat embryos. *Teratology* 50: 348-360.
- VAN STRAATEN, H.W.M., BLOM, H., PEETERS, M.C.E., ROUSSEAU, A.M.J., COLE, K.J. and SELLER, M.J. (1995). Dietary methionine does not reduce penetrance in curly tail mice but causes a phenotype-specific decrease in embryonic growth. *J. Nutr.* 125: 2733-2740.
- WEEKS, B.S. and KLEIN, N.W. (1989). The *in vitro* and *in vivo* embryo toxicity of antilaminin antibodies in the rat. *Am. J. Reprod. Immunol. Microbiol.* 19: 57-64.
- WEEKS, B.S., KLEIN, N.W., KLEINMAN, H., FREDRICKSON, T. and SACKETT, G.P. (1989). Laminin immunized monkeys develop sera toxic to cultured rat embryos and fail to reproduce. *Teratology* 40: 47-57.
- WILSON, J.G. (1973). *Environment and Birth Defects*. Academic Press, New York.