Successful Culture of Rat Embryos on Human Serum: Use in the Detection of Teratogens

Abstract. *Growth of head-fold-stage rat embryos cultured with human serum for 48 hours was enhanced by supplementation with glucose. Embryo growth (protein and DNA contents) varied with the source of the serum. Serum from 16 of 19 untreated subjects produced normal embryos. Serum from five subjects undergoing cancer chemotherapy and six subjects receiving anticonvulsants was either lethal or teratogenic.*

Procedures for the culture of early rat embryos in vitro have been refined in recent years largely through the efforts of New *et al.* (*1*). Rat embryos have even been cultured with serum from rats injected with cadmium chloride or cyclophosphamide in order to detect changes in serum teratogenicity (*2*). In an attempt to extend this approach to man, we cultured rat embryos on human serum supplemented with glucose. We found that these embryos could be used to detect teratogens when serum from patients receiving medication for the treatment of cancer or epilepsy was used as the culture medium.

Head-fold-stage rat embryos and a 48-hour culture period were used in studies by our laboratory and by New (*3*) to indicate that human serum is inferior to rat serum as a culture medium for rats. However, it has been reported that somite-stage rat embryos can be successfully cultured on human serum for 18 to 22 hours (*4*). This success with a short culture period, together with estimates of glucose consumption by cultured embryos (*5*) and the higher concentration of glucose in rat serum (1.8 mg/ml) compared to human serum (0.98 mg/ml), suggested to us that glucose limits the growth of rat embryos on human serum. We achieved optimal embryo growth in vitro when 2 to 3 mg of glucose was added per milliliter of human serum. With glucose supplementation, embryo protein and DNA accumulation after 48 hours increased three- and fivefold, respectively, compared to growth of embryos cultured on unsupplemented human serum (Fig. 1, a and b). In subsequent experiments, all serum samples were analyzed for glucose concentration by the glucose oxidase method (*6*) and were adjusted to a final concentration of 3 mg/ml. In addition, human serum was prepared as a culture medium by the procedures used for preparing rat serum, including immediate centrifugation after withdrawal (*7*), heat inactivation for 30 minutes at 56°C (*7*), sterile filtration through an HA-type Millipore filter, supplementation with streptomycin sulfate (0.66 mg/ml) and penicillin G potassium (0.0006 mg/ml), and 10 percent dilution (by volume) with sterile water (*2*). Glucose solution supplement replaced a comparable volume of dilution water.

To determine whether embryo growth and development varied if serum from different persons was used as the culture medium, serum samples were obtained from ten male subjects, ages 22 to 33, and nine female subjects, ages 20 to 25. They were nonsmokers, were not taking medication (including oral contraceptives), and were considered to be in good health. Six embryos were cultured on each serum sample from nine males and seven females, five embryos were cultured on serum from one male, and three embryos were cultured on each serum sample from two females. Embryos cultured on individual samples contained 42.8 to 117.3 μg of protein each and 5.8 to 13.7 μg of DNA. Analysis of variance (*8*) indicated that variability in growth among embryos cultured on serum from different individuals was significantly greater than variability among embryos cultured on the same serum sample [for protein content, F (58, 47) = 10.03 and 5.34 in male and female subjects, respectively ($P < .001$); for DNA content, F

Table 1. Protein and DNA contents and abnormality frequencies of head-fold-stage rat embryos cultured for 48 hours on serum from untreated subjects or from subjects receiving cancer chemotherapy or anticonvulsants. Nulliparous CD-strain female rats (Charles River), mated with CD-strain males, were killed after 9.5 days (the morning of a positive vaginal smear for sperm was considered 0.5 day) and the embryos were removed. Head-fold-stage embryos were selected for culturing. Yolk sacs and ectoplacental cones were kept intact, but Reichert's membranes were removed (*2*). Culture vessels (*11*) containing three embryos in 2 ml of medium were maintained at 37.5°C, rotated at 30 rev/min, and gassed with O_2 (5 percent), CO_2 (5 percent), and N_2 (90 percent) immediately and after 12 hours; with O_2 (20 percent), CO_2 (5 percent), and N_2 (75 percent) after 24 hours; and with O_2 (40 percent), CO_2 (5 percent), and N_2 (55 percent) after 36 hours (*2*). The embryos were separated from the extraembryonic membranes, rinsed in saline, placed overnight in 5 percent trichloroacetic acid, and homogenized. After centrifugation, the pellets were extracted with 95 percent ethanol chloroform and ethanol (1:3) for 15 minutes at 70°C, and again with 95 percent ethanol. The DNA was then extracted with 5 percent perchloric acid at 70°C for 20 minutes (*12*). The remaining protein precipitate was rinsed with 95 percent ethanol and dissolved in 1*N* NaOH. Diphenylamine was used for DNA quantitation (*13*) and the method of Lowry *et al.* (*14*) for protein.

Subject	Sex of subject	Number of embryos cultured	Number of abnormal embryos	Protein (μg per embryo ± S.E.)	DNA (μg per embryo ± S.E.)
Untreated					
1 through 10	M	59	6	79.5 ± 6.6	9.9 ± 0.7
11 through 19	F	48	14	72.5 ± 5.8	9.8 ± 0.7
Chemotherapy					
20[a]	M	3	Lethal	10.5[b]	0.7[b]
21[c]	M	3	Lethal	9.1[b]	0.6[b]
21 (after treatment)	M	3	0	81.7 ± 2.9	10.5 ± 0.6
22[d]	M	3	0	86.6 ± 9.1	7.7 ± 0.6
23[e]	F	3	Lethal	20.6[b]	0.7[b]
24[f]	F	3	3	54.4 ± 1.7	3.5 ± 0.6
25[g]	F	3	3	88.5 ± 2.0	4.6 ± 1.0
Anticonvulsant					
26[h]	M	3	3	63.8 ± 4.8	8.6 ± 0.4
27[i]	M	3	3	60.8 ± 4.0	5.9 ± 1.1
28[j]	M	3	3	13.8[b]	4.2[b]
29[k]	M	3	3	83.0 ± 1.3	9.6 ± 0.4
30[l]	M	3	3	79.3 ± 6.6	8.6 ± 0.4
31[m]	F	3	3	71.9 ± 1.2	8.9 ± 0.4

[a]Subject 20 received vincristine (1 mg intravenously every 3 weeks) and Lomustine (100 mg orally every 6 weeks) and was given vincristine on the day of sampling. [b]Embryos were pooled for analysis of protein and DNA content; therefore standard errors (S.E.) are not calculated. [c]Subject 21 received vincristine (2 mg intravenously every 3 weeks), Adriamycin (30 mg intravenously every 3 weeks), Lomustine (100 mg orally every 6 weeks), and cyclophosphamide (1 g orally every 6 weeks) and was given vincristine and Adriamycin on the day of sampling. [d]Subject 22 received no chemotherapy but was diagnosed as having cancer and, therefore, is included here for comparison. [e]Subject 23 received cyclophosphamide (100 mg per day, orally). [f]Subject 24 received cyclophosphamide (100 mg per day, orally), methotrexate (75 mg intravenously every 4 weeks), and 5-fluorouracil (1.5 g intravenously every 4 weeks). [g]Subject 25 received tamoxifen (20 mg per day, orally). [h]Subject 26 received Tegretol (400 mg per day, orally). [i]Subject 27 received Peganone (500 mg per day), Tegretol (1.2 g per day), Primidone (875 mg per day), and Depakene (1.25 g per day), all given orally. [j]Subject 28 received Primidone (750 mg per day), carbamazepine (800 mg per day), and Depakene (1.5 g per day, all given orally). [k]Subject 29 received Tegretol (800 mg per day), Mellaril (250 mg per day), and Dilantin (350 mg per day), all given orally. [l]Subject 30 received Tegretol (800 mg per day), Depakene (1 g per day), and Primidone (500 mg per day), all given orally. [m]Subject 31 received Dilantin (300 mg per day), orally.

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(58, 47) = 5.09 and 12.60 for males and females, respectively ($P < .001$)]. The average protein and DNA contents of embryos cultured with serum from males did not differ significantly from those of embryos cultured on female serum. In comparison to previous data from this laboratory (*2*), the average protein content of embryos cultured on human serum was 73 percent of that of embryos cultured on rat serum, and the DNA content, 117 percent. Histological comparison between embryos cultured on rat serum and glucose-supplemented human serum showed comparable morphological development (Fig. 1, c and d). The embryos had closed neural tubes, 21 to 26 somite pairs, forelimb buds, otocysts, and three pairs of aortic arches and branchial pouches. When cultured with serum from males, 6 of 59 embryos developed abnormally; when cultured with serum from females, 14 of 48 embryos showed abnormalities. However, serum from just one male subject accounted for five of the six abnormal embryos that had been cultured on male serum, and serum from only two female subjects accounted for 12 of the 14 abnormal embryos that had been cultured on female serum. Abnormal embryos contained less protein and DNA than normal embryos produced on serum from untreated individuals; body curvature was incomplete; and neural tubes were exencephalic (had failed to close at several points).

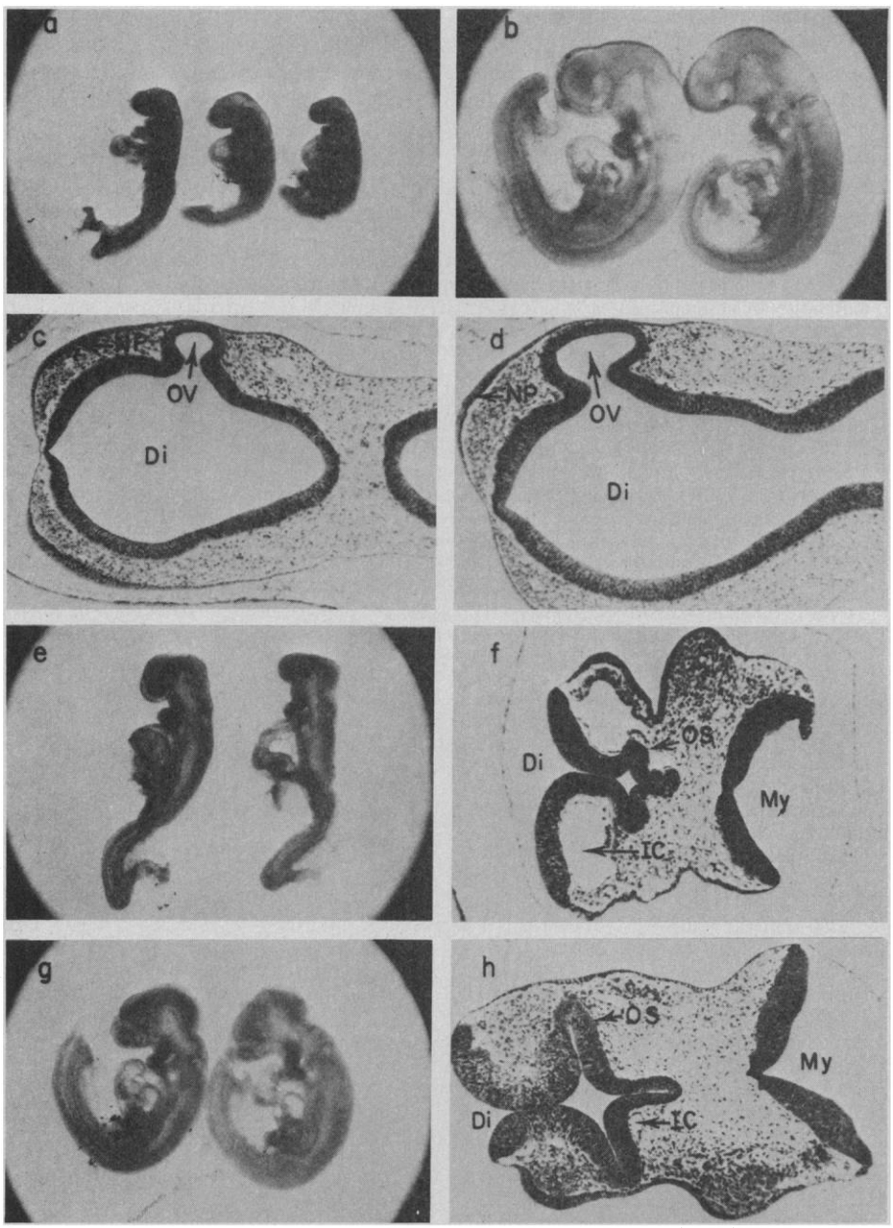

Fig. 1. Head-fold-stage rat embryos after 48 hours of culture on various serums. Live embryos were either photographed immediately after removal of extraembryonic membranes or placed in Carnoy's fixative with their membranes intact for sections. After routine processing, 7-μm paraffin sections were cut and stained with Ehrlich's hematoxylin and periodic acid-Schiff reagent. Whole embryos were photographed at ×18 and sections at ×42. (a) Embryos cultured on human serum without added glucose. (b) Embryos cultured on human serum supplemented with glucose (3.0 mg/ml). (c) Section of embryo cultured on rat serum. (d) Section of embryo cultured on human serum supplemented with glucose (3.0 mg/ml). (e and f) Embryos and section after being cultured with serum from subject 24, who was undergoing chemotherapy for cancer. (g and h) Embryos and section after being cultured with serum from subject 27, who was receiving anticonvulsants. Abbreviations: *Di*, diencephalon; *My*, myelencephalon; *OV*, optic vesicles; *OS*, optic sulcus; *NP*, nasal placode; and *IC*, internal carotid artery.

To test the feasibility of using cultured rat embryos to detect teratogens in human serum, serum donors were selected who were receiving continuous drug treatment with known or suspected teratogens. Serum from three subjects who were undergoing cancer chemotherapy was lethal to embryos during the first 24 hours of culture (Table 1). When these serum samples were diluted with untreated human serum (1:1 by volume), they remained lethal. Serum from one subject was still lethal when diluted 1:3 (by volume) with untreated human serum. Serum from two other chemotherapy subjects allowed embryo survival for 48 hours, but all the embryos were abnormal and protein/DNA ratios were higher than ratios for untreated subjects [ratios for untreated subjects ranged from 6 to 10; ratios for two treated subjects (Nos. 24 and 25) were 15 and 19]. Abnormalities among these surviving embryos included retarded body curvature, absence of limb buds (Fig. 1e), exencephaly, dilation of the internal carotid artery, retarded eye development (Fig. 1f), and reduced numbers of somites (16 to 18 pairs), aortic arches (one pair), and branchial pouches (two pairs).

It was possible to obtain serum samples from one subject (No. 21) both during chemotherapy and 3 months after stopping chemotherapy. Serum obtained during treatment was lethal to embryos, whereas serum obtained after treatment supported growth comparable to that of normal embryos cultured with serum from untreated subjects (Table 1). When serum from a subject (No. 22) diagnosed as having cancer but not receiving chemotherapy was used, embryos also developed normally.

All embryos survived 48 hours of culture in serum from six subjects who were receiving anticonvulsant treatment, but all were exencephalic and failed to develop optic vesicles (Fig. 1h). Embryos cultured on serum from one of these subjects were extremely small and showed extensive tissue necrosis. All other em-

bryos were comparable to normal embryos cultured on serum from untreated subjects with respect to body curvature, forelimb buds, pharyngeal development, and nasal placodes (Fig. 1g). Somite pairs ranged in number from 17 to 24. Protein and DNA values for embryos cultured in four of the six serum samples did not differ significantly from those obtained for embryos cultured with serum from untreated subjects (Table 1).

Thus we demonstrated that growth of rat embryos in human serum can be appreciably enhanced by glucose supplementation and that growth and development varies with the individual source of the culture serum. Indeed, we have observed death and abnormalities in rat embryos cultured with serum from humans suffering from colds and with serum from rats suspected of having pneumonia.

Culture medium prepared from serum from persons who were receiving drugs that are teratogenic to animals (*9*) or implicated as teratogenic to humans (*10*) was lethal or teratogenic to rat embryos. Although this demonstrates that cultured rat embryos can be used to detect teratogenic substances, no attempt was made to characterize the deleterious substances in the serum samples. Such an analysis is required to establish a relation between the effects of human serum on rat embryos in vitro and the occurrence of congenital defects in man.

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References and Notes

1. D. A. T. New, P. T. Coppola, D. L. Cockroft, *J. Embryol. Exp. Morphol.* **36**, 133 (1976).
2. N. W. Klein, M. A. Vogler, C. L. Chatot, L. J. Pierro, *Teratology*, in press.
3. D. A. T. New, *J. Reprod. Fertil.* **12**, 509 (1966).
4. T. Tanimura and T. H. Shepard, *Proc. Soc. Exp. Biol. Med.* **135**, 51 (1970).
5. D. L. Cockroft and P. T. Coppola, *Teratology* **16**, 141 (1977).
6. H. U. Bergmeyer and E. Bernt, in *Methods of Enzymatic Analysis*, H. U. Bergmeyer, Ed. (Academic Press, New York, 1974), p. 1205.
7. C. E. Steele and D. A. T. New, *J. Embryol. Exp. Morphol.* **31**, 707 (1974).
8. R. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics* (McGraw-Hill, New York, 1960).
9. The chemotherapy drugs used in this study and shown to be teratogenic to animals were Adriamycin [D. J. Thompson, J. A. Molello, R. J. Strebling, I. L. Dyke, *Teratology* **17**, 151 (1978)], Lomustine [_____, *Toxicol. Appl. Pharmacol.* **34**, 456 (1975)], cyclophosphamide [N. W. Klein, M. A. Vogler, C. L. Chatot, L. J. Pierro, *Teratology*, in press; R. D. Short, K. S. Rao, J. E. Gibson, *ibid.* **6**, 129 (1972)], 5-fluorouracil [J. G. Wilson, R. L. Jordan, H. Schumacher, *ibid.* **2**, 91 (1969)], methotrexate [R. L. Jordan, J. G. Wilson, H. J. Schumacher, *ibid.* **15**, 73 (1977); R. G. Skalko and M. P. Gold, *ibid.* **9**, 159 (1974)], tamoxifen [J. A. Furr, B. Valcaccia, J. R. G. Challis, *J. Reprod. Fertil.* **48**, 367 (1978)], and vincristine [V. H. Ferm, *Science* **141**, 426 (1963)]. Anticonvulsants so shown and used in our study were Dilantin and Primidone [R. D. Harbison and B. A. Becker, *Teratology* **2**, 305 (1969); *Toxicol. Appl. Pharmacol.* **22**, 193 (1972); F. M. Sullivan, *Teratology* **8**, 239 (1973)].
10. The chemotherapy drugs used in this study and implicated as teratogenic to humans were cyclophosphamide [L. H. Greenberg and K. R. Tanaka, *J. Am. Med. Assoc.* **188**, 423 (1964)] and methotrexate [A. Milunsky, J. W. Graef, M. F. Gaynor, *J. Pediatr.* **72**, 790 (1968); G. T. Ross, *Cancer* **37**, 1043 (1976)]. The anticonvulsant drug so implicated and used in our study was Dilantin [J. W. Hanson and D. W. Smith, *J. Pediatr.* **87**, 285 (1975); J. W. Hanson, N. C. Myrianthopoulos, M. A. S. Harvey, D. W. Smith, *ibid.* **89**, 662 (1976); R. R. Monson, L. Rosenberg, S. C. Hartz, S. Shapiro, O. P. Heinonen, D. Slone, *N. Engl. J. Med.* **289**, 1049 (1973); T. S. Yang, C. C. Chi, C. J. Tsai, M. J. Chang, *Obstet. Gynecol.* **52**, 682 (1978)]. For reviews, see J. F. Annegers, L. R. Elveback, W. A. Hauser, L. T. Kurland, *Arch. Neurol.* **31**, 364 (1974); Y. Biale, H. Lewenthal, N. B. Anderet, *Obstet. Gynecol.* **45**, 439 (1975); J. Fedrick, *Br. Med. J.* **2**, 442 (1973); R. M. Hill, W. M. Verniaud, M. G. Horning, L. B. McCulley, N. F. Morgan, *Am. J. Dis. Child.* **127**, 645 (1974); S. R. Meadow, *Proc. R. Soc. Med.* **63**, 12 (1970).
11. N. W. Klein, P. P. Minghetti, S. K. Jackson, M. A. Vogler, *J. Exp. Zool.* **203**, 313 (1978).
12. J. M. Webb and H. V. Lindstrom, *Arch. Biochem. Biophys.* **112**, 273 (1965).
13. K. W. Giles and D. A. Myers, *Nature (London)* **206**, 93 (1965).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
15. We thank J. D. McLaughlin, A. W. Zimmerman, and J. S. Nystrom of the University of Connecticut Health Services and School of Medicine for supplying serum samples and F. Jordan of Environmental Teratology Information Center for a literature search. Scientific contribution 765, Storrs Agricultural Experiment Station, University of Connecticut. This work was supported by DOE contract EY-76-S-02-3139.A003 (Office of Health and Environmental Research).

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Dendrodendritic Inhibition: Demonstration with Intracellular Recording

Abstract. *The isolated turtle olfactory bulb was used to characterize synaptic interactions between mitral and granule cell dendrites. First, blockade of antidromic invasion of mitral cell dendrites caused a large decrease in the size of the inhibitory postsynaptic potential (IPSP) recorded in mitral cells, indicating that the IPSP results in large part from activity in the dendrites of mitral cells. Second, direct depolarization of mitral cells was followed by an IPSP. In the presence of tetrodotoxin, depolarization of mitral cells evoked calcium spikes (as would be expected for presynaptic membranes) followed by IPSP's. These findings demonstrate that regenerative sodium spikes and axonal pathways are not required for this inhibitory pathway and that the inhibition is indeed a result of local dendritic interaction. Such a pathway provides an obligatory synaptic inhibition of mitral cells following their activation and emphasizes the tight coupling that exists with reciprocal dendrodendritic synapses.*

Local circuits of the central nervous system (CNS) are crucial components in the integration of sensory input (*1*). A particularly important example of local neuronal action is provided by dendrodendritic synaptic inhibition, which is likely to occur at numerous sites in the CNS. The specific properties of this synaptic interaction are still poorly understood, however, and, except for the olfactory bulb, the evidence is limited almost entirely to anatomical findings. In the olfactory bulb, the physiological evidence is based largely on the analysis of extracellular field potentials with some supporting intracellular data (*2*). In the bulb (Fig. 1A) it is envisaged (*2*) that depolarization of mitral cells and their dendrites activates excitatory synapses which depolarize granule cell dendrites. This depolarization in turn activates inhibitory synapses made by granule cells that release γ-aminobutyric acid (GABA) back onto the same, and neighboring, mitral cells. This pathway has also been proposed to be activated by graded electrotonic depolarization. A number of predictions can be made: (i) calcium channels should exist in mitral cell soma-dendrites since these cells are presynaptic; (ii) depolarization of mitral cells should result in a local self-inhibition that is independent of axonal pathways; (iii) the inhibitory pathway should remain intact in the absence of action potentials; (iv) the inhibition of a mitral cell should be greater when an antidromic impulse invades the soma-dendritic membrane than when the impulse is limited to the axon and initial segment of the mitral cell. We have used the isolated turtle olfactory bulb (*3*) to test these predictions.

Turtles (*Pseudemis scripta elegans*) were decapitated, and the olfactory bulb was removed and placed in Ringer solution (*4*). The bulb was hemisected to facilitate the placement of electrodes. We used a bipolar concentric electrode to stimulate the olfactory nerves and a bipolar needle electrode to stimulate the mitral cell axons antidromically. Microelectrodes (100 to 200 megohms) filled with 2*M* potassium methyl sulfate or 3*M* potassium chloride were used for intracellular recording. A bridge circuit was used for passing current through the recording electrode. Switching between

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