NEURAL ABNORMALITIES INDUCED BY SELECTED CHEMICAL AGENTS

Phillip L. Bressman and Frank Seto

Department of Zoology, University of Oklahoma, Norman, Oklahoma

Four drugs— trypan blue, urethane, cyclophosphamide and methotrexate— were tested to ascertain their deleterious effects on the neural development of the mouse embryo. The drugs were administered i.p. into pregnant females on the 7th to 9th day of gestation. The animals were sacrificed on the 14th day and gross and microscopic examinations of the uterine contents were made. From counts of viable fetuses and resorption sites all four drugs appeared to increase fetal mortality, methotrexate the most, and urethane the least. Within the range of the drug dosage tested only cyclophosphamide treatment resulted in grossly visible neural defects; however, microscopic examination of representative fetuses in the trypan blue and urethane treatment groups revealed histologically obvious degenerative alterations. Methotrexate-treated fetuses appeared to be unaffected.

INTRODUCTION

Mammalian development requires a multitude of sequential, genetically ordered developmental steps before the individual is fully formed so that it is prone to intrinsic congenital malfunctions (1) and is susceptible to interference by various extrinsic agents (2). Among the nongenetic agents that can adversely alter the development of the mammalian embryo or fetus are potentially scores of chemicals. Laboratory screening experiments have shown that many chemicals have malignant effects on the unborn (3), and interest in chemical teratology has expanded greatly in recent years.

The dye trypan blue, a classic vital stain (4); urethane, once used as a pain reliever and sedative (5) but now an ingredient in pesticides, fumigants and cosmetics; cyclophosphamide, an alkylating agent, and methotrexate, a folic acid antagonist, both currently used as effective anticancer agents (6), are all teratogenic as well (7,8, 9, 10). The four drugs were tested to ascertain their specific detrimental effects on the neural development of the mouse embryo. The 7th to 12th day of gestation is a particularly sensitive period. At this time the neural plate has formed and neural morphogenesis is at its height and should be especially susceptible to the action of teratogens. The experimental results indicate that the four chemicals at the dosage tested have different detrimental effects on the mouse embryo.

MATERIALS AND METHODS

Subjects

A total of 139 Swiss albino mice (127 females and 12 males) were used for these experiments. Breeding groups consisted of six females and two males in a cage and the females were examined periodically in the morning and afternoon for evidence of mating. Females with fresh vaginal plugs were isolated from the group and the date recorded as the first day of pregnancy as have been done by others (11).

Administration of Drugs

The seventh day of pregnancy was chosen as the time to begin the treatments. Except for cyclophosphamide, the injection procedures were similar to those reported in the literature (5, 12, 13).

An aqueous solution of trypan blue was prepared in advance and stored in the refrigerator for periods up to two weeks prior to use. Injections of ¼ ml of 0.4% trypan blue were administered on days 7, 8, and 9.

Urethane was freshly dissolved in sterile distilled water. Injections of ½ ml of a 3% urethane solution were given to pregnant mice on days 7 and 9.

Cyclophosphamide (Cytoxan®) was dissolved in distilled water and a single ½-ml injection of the 0.12% solution was given

immediately to females, seven days into pregnancy.

A 0.12% methotrexate solution was prepared in 2% $NaHCO_3$ and ½ ml injected into female mice on day 7.

All drugs were administered i.p. under semisterile conditions. Other pregnant females injected with saline or 2% $NaHCO_3$ solutions served as controls.

Collection and Examination of Fetuses

On the fourteenth day of pregnancy, about a week after the initiation of the drug treatments, the females were killed by over-exposure to ether and the uteri removed. These were examined with an illuminated magnifier or dissecting microscope for numbers of implantation sites, signs of resorptions, and viable fetuses. Each fetus was closely scrutinized for grossly visible malformation and fixed in Bouin's fluid for histological preparations. Selected fetuses were processed in paraffin, 10-micron sections cut, and these stained with hematoxylin and eosin. A set of control slides of normal mouse embryos of comparable age was also available for comparison. The sections were examined for histologically detectable deviations from the normal.

RESULTS

Observations of control females

The uterine contents of seven untreated or sham-treated pregnant females were examined and served as the control group. The numbers of implantation sites, viable fetuses, and resorption sites of the group are summarized in Table 1. Except for hematoma formation observed in four fetuses from females injected with 2% $NaHCO_3$ solution, no gross abnormalities were detected among the fetuses.

Frequency of embryo lethality and gross abnormalities

A summary of the examination of the uterine contents of pregnant females exposed to the four drugs is shown in Table 2. More resorption sites were observed among the experimental groups than the 6% in the control group. Successful implantations and viable fetuses were greatly reduced with methotrexate (28%), cyclophosphamide (58%), and trypan blue (62%) treatments and to a lesser extent with urethane (74%). Gross externally visible defects were generally not observed except in the case of cyclophosphamide, where microencephaly (abnormally small cerebral hemispheres) occurred in 3 of 21 viable fetuses. Multiple hematomas were found on 5 of 8 fetuses of the methotrexate group and 4 of 20 fetuses in the $NaHCO_3$-treated controls.

Microscopic examination of the fetuses

Representative fetuses from each experimental group were sectioned as previously described. They were compared with normal fetuses obtained from sham-treated or untreated control pregnant females. Figure 1 shows representative sections of a portion of the developing brain of a normal fetus (A) and those treated with cyclophosphamide (B), trypan blue (C), and urethane (D). Three fetuses from the methotrexate group were indistinguishable from the controls. The three microencephalic fetuses of the cyclophosphamide group were retarded in neural development but appeared otherwise to be histologically well differentiated. Four of five fetuses in the trypan blue group and one of the two fetuses of the urethane group, despite their superficially normal appearance, revealed obvious internal degenerative changes when examined microscopically. In the trypan blue group the basic neural organization was maintained but the tissues were clearly undergoing degenerative changes. The damage was more extensive in the brain region than posteriorly. The surrounding tissues and especially the mesenchyme were sparse. One fetus in the urethane group was normal and the other obviously morbid. In the latter the mesenchyme was overabundant and the cells were atypically rounded

TABLE 1. *Uterine contents of untreated and sham-treated control pregnant females.*

Group	Number of females	Implantation sites	Viable fetuses	Resorption sites
Untreated	2	17	17	0
Saline	3	29	27	2
Bicarbonate	2	22	20	2

and clumped. The cells of the neural epithelium were few in number and loosely and abnormally arranged. The neural epithelium was generally thin, often discontinuous, and thrown into numerous irregular folds.

DISCUSSION

Despite the limited scope of the experiments it is clear that the four drugs are detrimental to the embryos when administered to pregnant females during the sensitive period in the morphogenesis of the nervous system. Embryolethality, as measured by counts of viable embryos and resorption sites, was markedly greater among the drug-treated groups than the control group. Although grossly visible defects were observed only in the cyclophosphamide-treated fetuses, microscopic examinations of the superficially unaffected fetuses of the other drug-treated groups, however, revealed degenerative changes and abnormal histogenesis of neural tissues.

No gross defects were observed, however, with the trypan blue dosage used in our experiments. Histological examinations of the apparently normal fetuses revealed extensive cellular degeneration of the central nervous system. Trypan blue has been reported to induce neural malformations such as nonclosure of the neural folds, brain eversion, and eye defects in mice (12, 14), and hydrocephaly, spina bifida, exencephaly, and defective eyes in rats (15, 16, 17). Various other teratogenic effects have been reported in rabbits (7, 18), in chickens (19), and in amphibians (20). Although the mode of action of trypan blue is still unclear, the damage is not irreversible and substantial proportion of the affected embryos appear to recover from the effects (21).

Although the observation of the urethane-treated fetus is restricted to a single morbid case, microscopic post-mortem examination revealed a peculiar morphogenetic alteration of the neural system. The neural epithelium was poorly differentiated and thrown into numerous internal folds suggesting an exaggerated growth at the expense of histogenesis. Since urethane is known to have carcinogenic properties, the developmental abnormality was not unexpected. Exencephaly and other brain defects have been reported with this drug in mice (5, 22), in rats (23), and in hamsters (8).

Microcephaly was found in 14% of the fetuses of females exposed to cyclophosphamide. Similar anomalies have been described in mice exposed to the drug on the 10th to 12th day of gestation by others (9, 24, 25). Other teratogenic effects presumed to be elicited by cyclophosphamide have also been observed in rats (26, 27) and in humans (28).

The methotrexate-induced effects were not as easily evaluated because of high

TABLE 2. *Effects on the mouse fetus of treating pregnant females with trypan blue, urethane, cyclophosphamide, and methotrexate.*

Treatment group	Female number	Implantation sites	Viable fetuses	Resorption sites	Gross defects
I[a]	TB-1	9	6	3	None[b]
	TB-2	9	5	4	None
	TB-3	14	10	4	None
	TB-4	8	4	4	None
II[c]	U-1	5	0	3	Dead (two)
	U-2	9	9	0	None
	U-3	9	9	0	None
	U-4	8	5	3	None
III[d]	C-1	9	3	6	Microencephaly (two)
	C-2	8	0	8	None
	C-3	9	8	1	Microencephaly (one)
	C-4	10	10	0	None
IV[e]	M-1	9	8	1	Hematoma (five)
	M-2	11	0	11	—
	M-3	8	0	8	—

a. Treated with 3 injections of 0.4% trypan blue
b. None—no detectable gross abnormalities
c. Treated with 2 injections of 3% urethane
d. Treated with single injections of 0.12% cyclophosphamide
e. Treated with single injections of 0.12% methotrexate

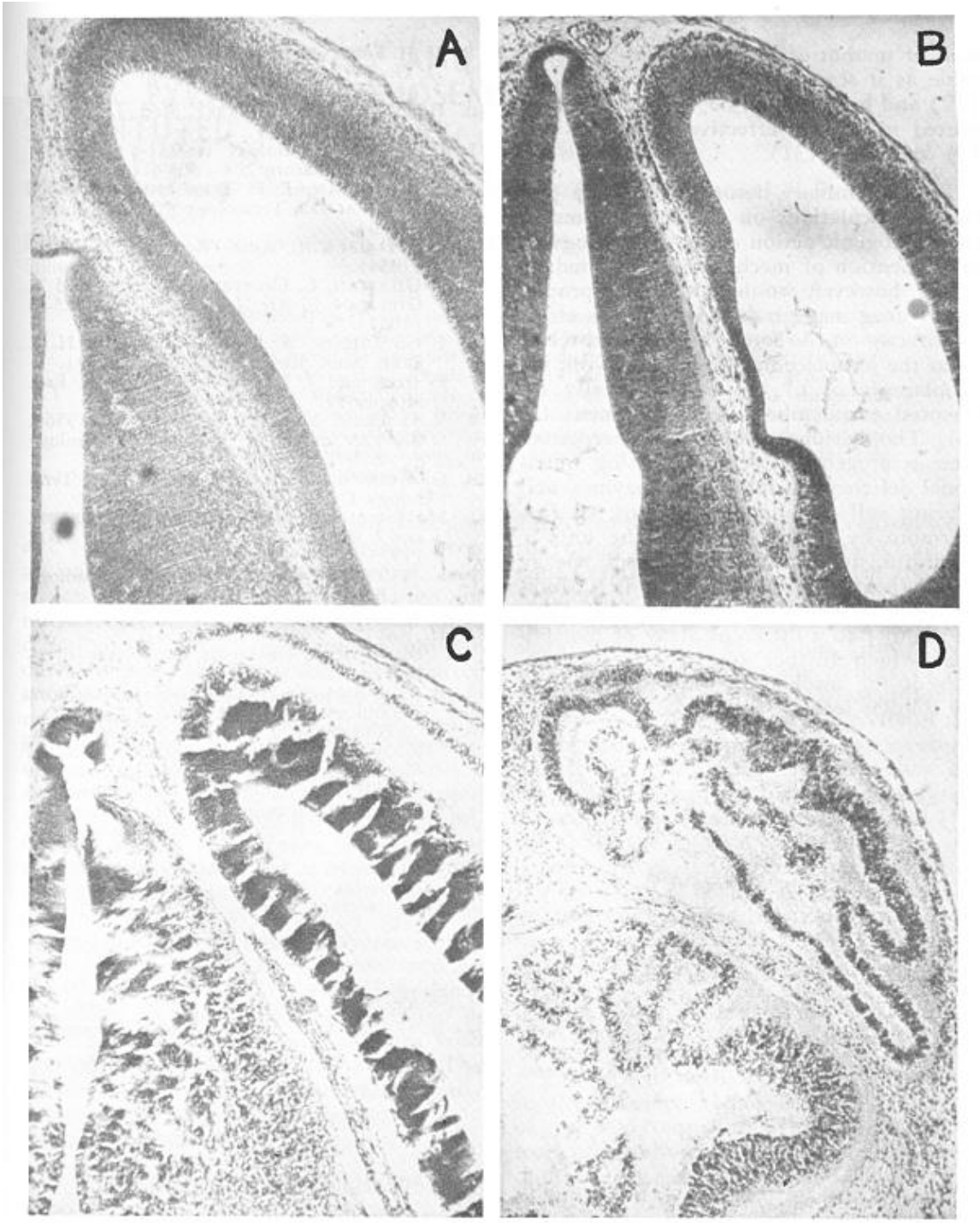

FIGURE 1. Representative quadrant sections of the developing brains of normal 14-day mouse fetus (A) and those exposed to cyclophosphamide (B), trypan blue (C), and urethane (D). Approximately similar brain areas of the different fetuses are shown at low power magnification.

embryolethality which resulted from the toxic dosage administered. Although no visible alterations were observed among the surviving fetuses, the possibility of detrimental effects on the nervous system cannot be excluded. The increased incidence of hematomas among the fetuses might be incidental, as these occurred in both methotrexate-treated mice and $NaHCO_3$-treated control groups. The teratogenic effective-

ness of methotrexate is not readily predictable as it seems to be teratogenic in rats (29) and humans (10, 30) but has been reported to be less effective in rabbits (7, 13) and mice (31).

The preliminary nature of the data precludes speculations on the mechanisms of the teratogenic action of the four drugs. A brief mention of mechanisms suggested by others, however, would not be inappropriate. A drug may produce its noxious effect a) directly on the fetus, or more indirectly b) at the fetoplacental site, i.e. the yolk sac or placenta or c) on the maternally influenced extra-embryonic environment (3, 32). The various drugs could perpetrate their teratogenic effects by causing nutritional deficiencies, inhibiting enzymes, and altering cell membrane functions, or furthermore by interfering with the mitotic apparatus, nucleic acid metabolism, energy metabolism, and osmolar balance (2). Moreover, the various drugs may be modified by the mother to a different array of metabolites, which further complicates the analysis of the mechanism of drug action at the cellular level.

REFERENCES

1. LAIRD JACKSON in: J. Lash and J. R. Whittacker (eds.), *Concepts of Development,* Sinauer Associates Inc., Stamford, Conn., 1974, pp. 380-403.
2. J. G. WILSON, Amer. J. Anat. 136: 129-32 (1973).
3. H. TUCHMANN-DUPLESSIS, Teratology 5: 271-86 (1972).
4. M. HAMBURGH, Nature 169: 27 (1952).
5. J. C. SINCLAIR, Texas Rep. Biol. Med. 8:623-32 (1950).
6. S. CHAUBE and M. L. MURPHY, Adv. Teratol. 3: 181-237 (1968).
7. C. E. ADAMS, M. F. HAY, and C. LUTWAKMANN, J. Embryol. Exp. Morph. 9: 468-91 (1961).
8. V. H. FERM, Arch. Path. 81: 174-77 (1966).
9. D. O. E. GEBHARDT, Teratology 3: 273-77 (1970).
10. H. POWELL and H. EKERT, Med. J. Aust. 2: 1076-77 (1971).
11. H. KALTER, Teratology 1: 231-4 (1968).
12. S. L. BECK, Nature 204: 403-4 (1964).
13. R. L. JORDAN, J. F. TERAPANE, and H. J. SCHUMACHER, Teratology 3: 203 (abstr.) (1970).
14. M. HAMBURGH, Anat. Rec. 119: 409-27 (1954).
15. J. GILLMAN, C. GILBERT, I. SPENCE, and T. GILLMAN, S. Afr. J. Med. Sci. 16: 125-35 (1951).
16. J. G. WILSON, A. R. BEAUDOIN, and H. J. FREE, Anat. Rec. 133: 115-28 (1959).
17. F. BECK and J. B. LLOYD, J. Embryol. Exp. Morph. 11: 175-84 (1963).
18. V. H. FERM, Anat. Rec. 125: 745-9 (1956).
19. S. KAPLAN and E. M. JOHNSON, Teratology 3: 269-72 (1970).
20. G. GREENHOUSE and M. HAMBURGH, Teratology 1: 61-74 (1968).
21. M. HAMBURGH, M. ERLICH, G. NATHANSON, and I. PESTETSKY, J. Exp. Zool. 192: 1-12 (1975).
22. K. TUTIKAWA and Y. HARADA, Teratology 6: 123 (abstr.) (1972).
23. E. K. HALL, Anat. Rec. 116: 383-94 (1953).
24. H. KALTER, *Teratology of the Central Nervous System*, University of Chicago Press, Chicago, 1968.
25. R. D. SHORT, K. S. RAO, and J. E. GIBSON, Teratology 6: 129-37 (1972).
26. D. NEUBERT, H. J. MERKER, E. KOHLER, R. KROWKI, and H. J. BARRACH, in Gerhard Raspe, ed. Advances in the Biosciences 6: Schering Symposium on Intrinsic and Extrinsic Factors in Early Mammalian Development, Pergamon Press, New York, 1971, pp. 575-622.
27. A. K. SANYAL, S. SINGH, and B. RAJU, Teratology 10: 95-6 (abstr.) (1974).
28. L. GREENBERG and K. R. TANAKA, J. Am. Med. Assoc. 188: 423-6 (1964).
29. J. G. WILSON, Anat. Rec. 166: 398 (abstr.) (1970).
30. A. MILUNSKY, J. W. GRAEF, and M. F. AYNOR, J. Pediatr. 72: 790-5 (1968).
31. R. G. SKALKO and M. P. GOLD. Teratology 9: 159-64 (1974).
32. F. M. SULLIVAN, Proc. Roy. Soc. Med. 63:1252-3 (1970).