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EMBRYOTOXIC ACTION OF CYCLOPHOSPHAMIDE AFTER BIOTRANSFORMATION IN CULTURES OF POSTIMPLANTATION RAT EMBRYOS

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The rapidly increasing number of pharmacological agents and also of chemical compounds polluting the environment makes the development of highly sensitive test systems for the detection of potential teratogens a particularly urgent task [1]. The use of cultures of postimplantation embryos of laboratory rodents for these purposes is spreading in the practice of research workers studying problems in applied teratology [4, 8]. However, the method of testing teratogens used in such cases, by direct addition of the test substances to the culture media [4, 6, 8, 11], can be used to detect the embryotoxic properties of the substances themselves, but not of their possible metabolic products.

Many workers are currently engaged on a search for approaches to the study of the embryotoxicity of metabolic conversion products of chemical substances formed directly in culture media to which either components of liver enzyme systems [7, 10] or cells of mammalian embryos [10] are added.

The object of this investigation was to study the possibility of using the microsomal fraction (MF) of rat liver and the essential cofactor NADPH [5] to bring about biotransformation of the known antitumor agent cyclophosphamide (CP) to teratogenic products in cultures of postimplantation rat embryos and to compare the sensitivity of this test system with that of the generally adopted method of studying embryotoxicity in pregnant animals [3].

EXPERIMENTAL METHOD

Experiments were carried out on embryos of 50 noninbred albino rats. On the 10th day of pregnancy the animals were killed by cervical dislocation and the uterus with embryos removed under sterile conditions. Embryos at the stage of formation of the 1st-3rd pairs of somites were cultured by New's method [12] at 37°C for 48 h in revolving (30 rpm) 100-cm^3 glass tubes containing 7.5 ml of homologous blood serum. For biotransformation of CP in vitro not more than 0.5 ml (total volume) of the necessary components were added to the incubation medium, so that their final concentrations in the medium, depending on the character of the experiment, were: protein of MF 0.4-2.0 mg/ml, NADPH 1 mM, CP 0.001-500 μg/ml. At the end of incubation

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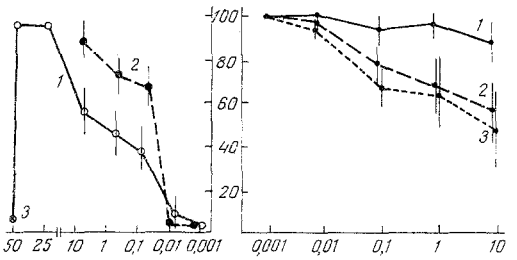

Fig. 1 Fig. 2

Fig. 1. Embryotoxic effect of CP in medium containing MF and NADPH. 1) Lethal action on embryos; 2) teratogenic action; 3) embryotoxic effect in medium without MF and NADPH. Abscissa, concentration of CP in medium (in μg/ml); ordinate, number of dying or abnormal embryos (in %).

Fig. 2. Indices of development of embryos in culture under the influence of different concentrations of CP in medium containing MF and NADPH. 1) Number of somites formed; 2) craniocaudal length; 3) total protein content. Vertical lines indicate mean error. Abscissa, concentration of CP in medium (in μg/ml); ordinate, values of parameters studied (in % of control).

the number of dead (absence of vitelline circulation) and abnormal embryos, the number of pairs of somites formed in the living embryos, the craniocaudal length, and total protein content were determined.

To obtain MF the liver of male rats was homogenized in cold phosphate–salt buffer (2 ml solution to 1 g tissue). MF was isolated under sterile conditions in the usual manner [2] and suspended in Hanks' solution in the ratio of 1 ml solution to 4 g of original tissue. The MF protein concentration in the suspension was about 30 mg/ml. The protein concentration in the MF suspension and embryos was measured by Lowry's method [9]. The suspension was poured into tubes and kept at –18°C. Activity of the MF used for biotransformation of CP was verified by determining the rate of p-hydroxylation of aniline by the cytochrome P-450 system [2].

In experiments in vivo CP in doses of 5-30 mg/kg was injected once, intraperitoneally, into female rats on the 10th day of pregnancy. Development of the embryos was analyzed on the 12th day, i.e., at a time corresponding to the end of the in vitro experiments.

EXPERIMENTAL RESULTS

The rate of p-hydroxylation of aniline in freshly isolated MF was 1.4-1.7 nmoles/mg protein/min. When frozen preparations of MF were kept for a long time, their p-hydroxylase activity fell in the course of 2 weeks on average by 33% and in a month by 55%. Accordingly, in the subsequent experiments MF preparations kept for not more than 2 weeks were used.

A study of the toxicity of MF for the embryos in culture showed that with an MF protein concentration in the incubation medium of 0.4-0.8 mg/ml development of the embryos was undisturbed (Table 1). The toxicity of higher concentrations of MF protein, expressed as death of the embryos and the formation of anomalies in living embryos (disturbance of neurulation along the whole length of the nerve tube), determined the limits of MF concentration used in the subsequent experiments, namely 0.4-0.8 mg/ml. Addition of NADPH to the culture medium containing MF did not affect development of the embryos (Table 1).

Without the addition of components necessary for its biotransformation CP caused death of 100% of explanted embryos only in concentrations of 250-500 μg/ml. In a concentration of 100 μg/ml it caused a decrease in the protein content in the embryos of 20% ($P < 0.05$). In a concentration of 50 μg/ml CP did not affect

TABLE 1. Embryotoxicity of Different Concentrations of MF Protein and Also of NADPH for Rat Embryos Developing in Culture

MF protein		NADPH·H_2		Total number of embryos	Number of dying embryos	Mean number of somites	Craniocaudal length, mm	Protein content, μg per embryo	Number of abnormal embryos
volume, ml	concentration, mg/ml	volume, ml	concentration, mM						
—	—	—	—	50	—	29,0±0,3	4,1±0,1	349±19	1
0,1	0,4	—	—	15	—	28,6±0,3	4,1±0,1	348±20	—
0,15	0,6	—	—	15	—	28,6±0,3	4,1±0,1	327±16	—
0,2	0,8	—	—	15	—	27,9±0,4	3,9±0,1	320±18	—
0,25	1,0	—	—	16	4	27,2±0.5	3,6±0,2	279±12	7
0,5	2,0	—	—	12	12	7—11	—	—	—
0,1	0,4	0,2	1,0	15	—	28,5±0,3	4,1±0,1	332±16	—
0,2	0,8	0,2	1,0	15	—	27,9±0,4	4,0±0,1	310±12	—

development of the embryos (Fig. 1). Experiments with bioactivation of CP were thus carried out at this and lower concentrations. The toxicity of the compound was found to increase sharply following its interaction with the enzyme system. For instance, in medium containing 25 and 50 μg/ml CPA and also NADPH and MF in concentrations not toxic for embryos, after only 12 h in culture all the embryos ceased to develop (Fig. 1). A further decrease in the CP concentration led to a decrease in the number of dying embryos, but most of those which remained alive were abnormal (Fig. 1). CP induced mainly reduction of the cerebral hemispheres with the onset of well-marked microcephaly, and also different types of disturbance of neurulation in the cranial portion. Another characteristic feature was a sharp decrease in the craniocaudal length of the body and in the total protein content, coupled with a generally insignificant decrease in the number of somites formed (Fig. 2). In medium containing MF and NADPH, CP continued to have an observable action down to a concentration of 0.1 μg/ml. If its concentration was reduced by a further one or two orders of magnitude, development of the embryos was unchanged and corresponded exactly to the control.

The study of the effect of CP on 10-day embryos showed that 48 h after exposure to the compound in doses of 15-30 mg/kg all the embryos were dead. Reducing the dose to 7.5 and 5 mg/kg led to a mortality of 31 and 9% respectively among the embryos, and developmental anomalies were found in 10 and 5% of embryos respectively. The character of the anomalies was the same as observed in experiments in vitro to study biotransformation of CP. This supports the view that in vitro, in the presence of MF and NADPH, activation of CP took place to teratogenic products, the same as which arise in vivo. A dose of 2.5 mg/kg did not affect embryos developing in utero.

To compare the sensitivity of the methods used, let the minimal acting doses (concentrations) of CP used in the experiments in vitro and in vivo be compared. If 1 ml of medium is conventionally taken to be equal to 1 g body weight of the animal, in experiments in vitro but without biotransformation of CP a toxic effect was reached at the level of 100 mg/kg, in the experiment with MF and NADPH in a dose of 0.1 mg/kg, and in experiments in which CP was administered to the mother, in a dose of 5 mg/kg. The high level of the acting dose and also the absence of a teratogenic effect in the first case undoubtedly reflect the toxicity of the CP itself in the absence of biotransformation to teratogenic products. The fact that identical deformities occurred in the 2nd and 3rd cases suggests a similar mechanism of action, connected with metabolic conversions of CP.

Detection of the products of teratogenic activation of CP, as used in these experiments, was thus found to be 50 times more sensitive than the method in vivo. It can be postulated that the use of a test system which includes culture of postimplantation rat embryos in conjunction with the microsomal fraction of the liver is promising for the study of the embryotoxicity of chemical substances undergoing metabolic conversions in vivo.

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EFFECT OF PHENFORMIN ON FETAL WEIGHT IN RATS PREVIOUSLY IMMUNIZED WITH HOMOLOGOUS THYROID GLAND TISSUE EXTRACT

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It was shown previously that the weight of fetuses of rats immunized at certain times before pregnancy with heterologous or homologous thyroid antigens is above the average value for this parameter in normal pregnant rats [4]. In rats immunized with homologous thyroid antigens, 2-6 weeks after the end of immunization lowering of the glucose tolerance, hyperplasia followed by hypertrophy of the islets of Langerhans in the pancreas, and moderate hyperinsulinemia with a blood insulin concentration 30-40% higher than initially [5] were observed (more marked hyperinsulinemia – a blood insulin level 100-400% higher than initially – was observed in rabbits immunized with thyroglobulin [11]). Injection of the antidiabetic biguanide phenformin during immunization of the rats and for 2-4 weeks thereafter abolished the disturbance of glucose tolerance and the hyperinsulinemia [5].

In the investigation described below the weight of the fetuses was determined in rats immunized with thyroid antigen and simultaneously treated with phenformin in order to determine whether the macrosomia of fetuses in animals subjected to this treatment can be prevented.

EXPERIMENTAL METHOD

Experiments were carried out on female rats from the Rappolovo nursery. The animals were immunized 4 times, at intervals of 1 week, with homologous thyroid antigen [4, 5, 9], divided into two groups: the rats of group 1, starting from the 1st day of immunization and until the 30th day after its end, received phenformin perorally by means of a tube in a dose of 7.5 mg in 1 ml water; the rats of group 2 received the same volume of water. The animals of group 3 were not immunized, but in the course of the experiment they were given 1 ml of water perorally by means of a tube. Mating of the animals began 1.5-2 months after the end of immunization (2-4 weeks after the end of administration of phenformin). The dating of the beginning of pregnancy and the weighing of the fetuses and placenta, removed 21.5 days (516 h) after that moment, were carried out as described previously [2]. The results were subjected to statistical analysis.

EXPERIMENTAL RESULTS

Preliminary immunization of the rats with homologous thyroid antigen led to a subsequent increase in the mean weight of the fetuses compared with those of normal pregnant rats (Table 1). Administration of phenformin during immunization and for 30 days after its end led to a significant decrease in the mean weight of the fetuses. No significant differences were observed in the increase in body weight during pregnancy and the weight of the placentas between the groups compared (Table 1). Phenformin had virtually no effect on the weight of the fetuses of the rats if the interval between the end of immunization and parturition was short (12 weeks) and it increased with an increase in the length of that interval (Fig. 1).

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