ONCOLOGY

Comparison of the Specific Binding of 17β-Estradiol with Receptor Proteins in the Cytosol Fraction of Uteruses of CBA and C57Bl/6 Mice in the Course of 1,2-Dimethylhydrazine-Induced Carcinogenesis

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The level of specific binding and affinity of 17β-estradiol for receptors in the cytosol fraction of uteruses of CBA and C57Bl/6 mice exposed to 1,2-dimethylhydrazine for a long time was studied. Estrogen receptors were studied by separating free and receptor-bound hormone with dextran-coated carbon. The theoretical number of sites of ligand binding with receptor protein and the level of free binding sites were shown to be higher in CBA mice sensitive to carcinogenesis induction in comparison with C57Bl/6 mice resistant to the carcinogen effect in both the experimental and control groups over the course of the experiment. The ligand affinity for receptor protein was more or less the same in all the groups.

Key Words: *mouse; uterus; estradiol; receptor proteins*

An experimental model - uterine sarcoma induced by 1,2-dimethylhydrazine (DMH) in mice - was obtained at the Department of Carcinogens, Cancer Research Center [17]. Studies revealed appreciable differences in the induction of this tumor. In CBA and C3H mice uterine sarcoma developed with a high frequency (40.7 and 37.5%), whereas in C57Bl/6 mice under the same conditions sarcoma developed extremely rarely (2.7%), and in C3HA mice it never developed [18]. Combined administration of DMH and estradiol dipropionate (EP) to female CBA mice accelerated tumor development [2], whereas pregnancy and progesterone inhibited this process [2,10]. The results indicate that EP, which is incapable of inducing uterine sarcoma by itself, markedly stimulates carcinogenesis in sensitive CBA mice when administered after carcinogen exposure [11].

Experiments with resistant C3HA animals demonstrated the possibility of inducing uterine sarcoma by combined exposure to DMH and EP, whereas neither of these agents induced sarcoma if used alone [12].

Study of the estral cycle showed DMH to be conducive to the development of persistent estrus in CBA mice sensitive to sarcoma induction and to estrus prolongation in the majority of resistant C57Bl/6 and C3HA mice [9].

In contrast to resistant C57Bl/6 and C3HA animals, in oophorectomized females of the CBA

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strain sensitive to sarcoma induction an injection of physiological doses of estrogen led to rapid enlargement of the uterus and to a much more rapid appearance of scales in the vagina. Hence, there are strain-specific differences in sensitivity not only to the carcinogen, but to estrogens as well [4]. Experiments on rats demonstrated that a single injection of DMH affects the neuroendocrine system by raising the threshold sensitivity to estrogens [6].

Estrogens are known to mediate their effect through specific receptors present in the cytosol fraction of uterine tissue. It was thus of interest to compare the levels of specific binding of 17β-estradiol with uterine receptors in females of different age, the ligand affinity for receptor proteins in the uterine cytosol, and the effect of multiple injections of DMH on the level of estrogen receptors in mouse strains sensitive (CBA) and resistant (C57Bl/6) to estrogens.

MATERIALS AND METHODS

Experiments were carried out with 15-week female CBA and C57Bl/6 mice bred at the Stolbovaya Breeding Center, Russian Academy of Medical Sciences. Four series of experiments were carried out, in which groups of animals of both strains administered carcinogens and age-matched intact controls were tested. Experimental mice were subcutaneously injected DMH dissolved in distilled water (pH 6-7) in a dose of 8 mg/kg body weight weekly according to the following protocols: first series 1 injection, second series 5, third series 11, fourth series 31 injections.

In the estral phase of the cycle detected by vaginal smears 6-12 animals from each group were sacrificed under ether narcosis 2 days after the last injection of DMH. Uteruses with tumors were not included in the experiment.

Estrogen receptors were detected by competitive binding with protein. The uterus was isolated, washed free of blood with 0.9% NaCl solution, cleared of fat, weighed, fractionated, crushed in a porcelain mortar with liquid nitrogen, and mixed with a 6-fold volume of cooled buffer containing 10 mM Tris-HCl (Merck), 1.5 mM EDTA (Sigma), 0.5 mM dithiotriiol (Koch-Light Laboratories), 0.03% sodium azide (Merck), pH 7.4, at 20°C, and 10% glycerol (ultra-pure) by volume. After defrosting the mixture was centrifuged for 10 min at 2500-3000 g. The cytosol was isolated from the supernatant by centrifugation of samples at 105,000 g for 1 h in a K-32M ultracentrifuge. The protein concentration in the cytosol was measured as described previously [16]. Cytosol with a total protein concentration of 1 to 2.0 mg/ml was used. 100 μl samples of cytosol were incubated for 14-16 h at 4-8°C with [1,2,6,7-^{3}H]-estradiol-17β ([^{3}H]-E_2) (Amersham). The preparation used contained at least 98% of [^{3}H]-E_2, purified as described previously [3]. Estradiol receptors were detected by separation of free and receptor bound hormone with dextran-coated carbon at a 5 nM saturating concentration of ligand with consideration of nonspecific binding in the presence of a 100-fold excess of diethylstilbestrol (Sigma). After the addition of the carbon suspension, the samples were shaken, left for 10 min at 0°C, and then centrifuged with cooling at 2500 g for 10 min. 200 βl supernatant were placed in flasks with 10 ml CZh-8 scintillation fluid. Radiometry of the samples was carried out using a Traucor Europa scintillator. Tritium scintillation efficacy was about 40%. Equilibrium dissociation constants (K_d) and the number of steroid molecules specifically bound to receptor proteins (specific binding sites) were determined after Scatchard at [^{3}H]-E_2 concentrations of 10^{-11}-10^{-8} mol/liter^{-1}.

The method of least squares was used to plot Scatchard's straight line diagram for each group of animals. The description of experimental points with a Scatchard plot was based on the determination coefficient (R^2). The plot was considered accurate at R^2>0.8. Free sites of estrogen binding to receptors were detected at a 5 nM saturating concentration of ligand and specific binding was estimated.

TABLE 1. Alteration of Equilibrium K_d in Relation to the Number of DMH Injections and the Age of CBA and C57Bl/6 Mice (10^{-9} mol/liter)

Group	Number of DMH injections			
	1	531	11	31
Age, weeks	15	19	25	45
CBA control	0.120±0.026	0.181±0.046	0.322±0.047	1.110±0.142
CBA experiment	0.148±0.016	0.433±0.052	0.588±0.067	1.510±0.159
C57Bl/6 control	0.158±0.042	0.149±0.026	0.399±0.093	0.353±0.075
C57Bl/6 experiment	0.093±0.015	0.192±0.047	0.250±0.046	0.694±0.029

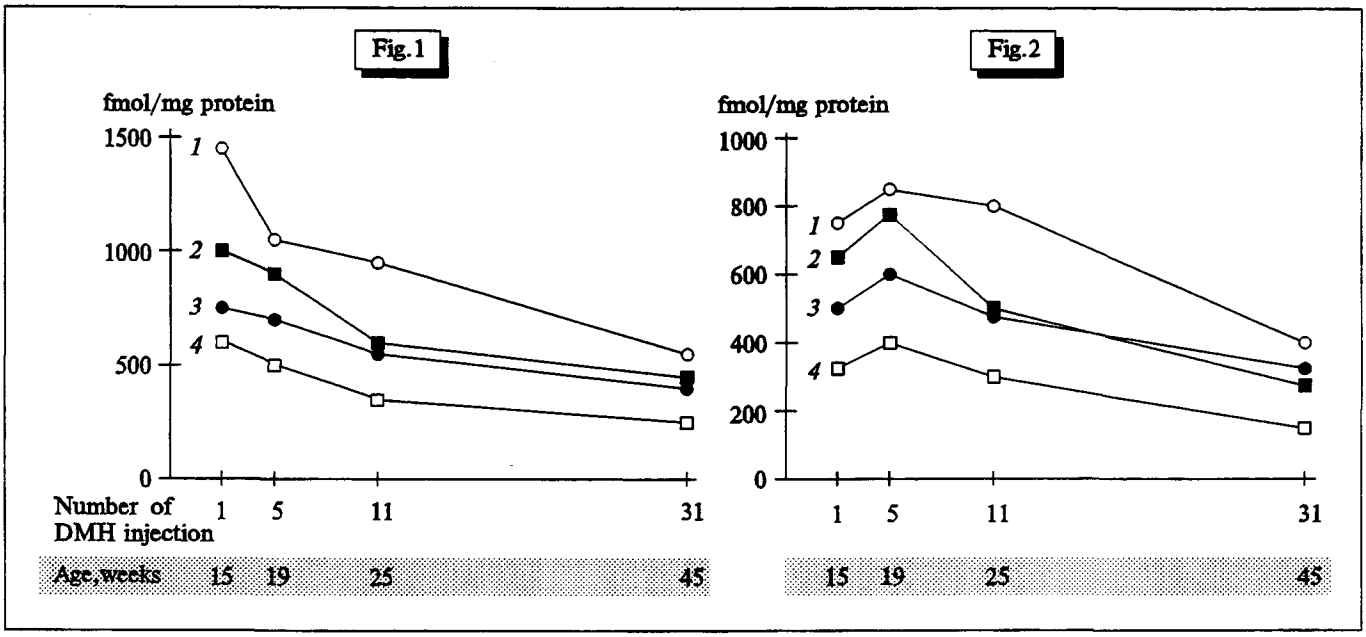

Fig. 1. Alteration of the theoretical number of binding sites of estradiol with receptor proteins in the uteruses of CBA and C57Bl mice of different age after repeated injections of DMH. Here and in Fig. 2: *1*) CBA, experiment; *2*) CBA, control; *3*) C57Bl, experiment; *4*) C57Bl, control.

Fig. 2. Changes in level of estrogen receptors (free sites) in the uteruses of CBA and C57Bl mice of different age after repeated injections of DMH.

RESULTS

We found no differences in the K_d value of [^{3}H]-E_2 with receptor proteins in the cytosol fraction of uterine tissue of 15-week-old females of the sensitive CBA and resistant C57Bl/6 strains (Table 1). A subsequent follow-up of control 19-, 25, and 45-week sensitive CBA mice showed a trend toward a reduction of [^{3}H]-E_2 capacity to bind with receptor protein in the uterine tissue cytosol, which was manifested by a K_d increase. Injection of DMH did not alter [^{3}H]-E^2 capacity to bind with receptor proteins in sensitive CBA or resistant C57Bl/6 mice. The K_d values were the same both in the experimental groups injected DMH and in the controls (Table 1). With aging, experimental animals showed the same tendency toward a reduction of [^{3}H]-E_2 capacity to bind with receptor proteins in the uterine tissue cytosol as did the intact females. Moreover, like the controls, experimental CBA mice showed a more marked tendency toward a reduction of the binding capacity in comparison with the resistant C57Bl/6 animals (Table 1).

The theoretical number of binding sites of the ligand with receptor protein in 15-week sensitive CBA mice was higher than in resistant C57Bl/6 animals (988 and 592 fmol/mg protein, respectively, Fig. 1). On the other hand, study of free estrogen receptors in the cytosol of murine uterine tissue at a saturating concentration of the ligand also revealed an increased level thereof in CBA in comparison with C57Bl/6 mice (660 and 347 fmol/mg protein, respectively, Fig. 2). With aging, the number of binding sites decreased both in sensitive CBA and in resistant C57Bl/6 mice (Fig. 1). However, the interstrain differences were observed till the end of the experiment, the number of binding sites being higher in the sensitive CBA mice. A negligible age-associated decrease in the levels of free receptors of estrogens was observed in animals of both strains only in the 25th week of life, but by the 45th week their number was less than half that during week 15 (Fig. 2). Nonetheless, the number of free receptors was higher in sensitive CBA mice than in resistant C57Bl/6 animals over the course of the experiment. A single injection of DMH caused an increase of the number of binding sites in CBA mice to 1452 fmol/mg protein (988 fmol/mg protein in the control) and to 744 fmol/mg protein in C57Bl/6 mice (592 fmol/mg protein in the control) (Fig. 1). After 5, 11, and 31 injections of DMH the number of binding sites was higher in injected mice than in the control (Fig. 1). At the same time, despite an increase of receptor level in resistant C57Bl/6 mice after DMH, their level was still lower than in sensitive CBA mice over the entire experiment (Fig. 1).

The theoretical number of binding sites gradually decreased with age in mice of both strains in-

jected DMH, similarly as in the control groups, but its value remained higher in experimental groups (Fig. 1).

The first injection of DMH resulted in an increase of the number of free estrogen receptors in the cytosol of uterine tissue of sensitive CBA mice (770 fmol/mg protein) *vs.* the control (660 fmol/mg protein) and the resistant C57Bl/6 strain (504 fmol/mg protein in the experiment and 340 fmol/mg protein in the control). The level of free binding sites remained consistently high in experimental groups of both strains after the 5th and 25th injections of DMH and dropped after the 31st injection of the carcinogen. However, the number of free receptors in the cytosol of uterine tissue of CBA mice injected DMH was higher over the whole experiment than in C57Bl/6 mice administered the same DMH dose. Moreover, the level of free estrogen receptors was higher in intact CBA mice than in experimental C57Bl/6 animals.

Table 1 shows that [^{3}H]-E_2 K_d with receptor proteins in the uterine tissue cytosol and, hence, [^{3}H]-E_2 capacity to bind with receptor proteins were characterized by values of the same order in both experiment and control after the first, fifth, and 25th injections. A lowered binding capacity was observed in the 45th week of life in control and experimental (after 31 injections of DMH) CBA mice. Apparently, this is a characteristic feature of this strain, which might be due to the earlier onset of age-associated changes in the endocrine status of CBA mice.

The level of estrogens in the blood and level of estrogen receptors in the rat uterine tissue cytosol are known to be in inverse correlation. The level of cytoplasmic estrogen receptors in the uterus drops as the estrogen level rises in the blood of female rats during sexual maturation, in old animals with persistent estrus, and after estrogen administration [5,7,8,15]. Conversely, a falling estrogen level in the blood, e.g., after oophorectomy, is associated with an increase of estrogen receptors [5,8].

The age-related reduction in the number of binding sites and free receptors in the uterine tissue cytosol of intact and experimental females of both strains correlates with published data on age-associated changes in the level of estrogen receptors in rats [8].

Previous studies revealed persistent estrus and irregular cycles with a prolonged estrus phase in CBA females under the effect of DMH as early as after the fifth injection, whereas in C57Bl/6 females persistent estrus was observed only after the 15th injection of DMH [9]. Moreover, a reduction in the level of cytoplasmic receptors was observed in old rats with persistent estrus [7]. Experiments with female rats revealed a higher threshold sensitivity of the hypothalamo-hypophyseal system to the homeostatic effect of estrogens after DMH injection [6]. Hence, multiple injections of DMH could be expected to reduce the levels of both free estrogen receptors and the theoretical number of binding sites. However, nothing of the kind was observed, and these values observed after the first, fifth, 25th, and 45th injections of DMH were higher than in the control (Figs. 1, 2). The DMH-boosted increase of the level of estrogen receptors is not a result of a rise of the level of endogenous estrogens, because an increase of the estrogen level and alteration of cycles with a prolonged estrus phase cause the level of estrogen receptors to drop. The effect of DMH on protein synthesis in the cell is evidently indirect. Hence, uterine tissue becomes more sensitive to estrogens.

Some other scientists have also observed changes in the levels of receptors of sex steroid hormones in target tissues of hormone-dependent organs in the course of carcinogenesis, but such reports are few. Ionizing radiation exposure was found to boost the level of estrogen receptors in mammary tissue of rats [14]. An increased content of androgen receptors was observed 5 months after methylcholanthrene treatment of the larynx in rats, in comparison with intact animals [1].

As mentioned above, there are strain-specific differences in the development of uterine sarcoma induced by DMH in mice and in the sensitivity of the uterus to estrogen. A much higher number of estrogen receptor binding sites in CBA mice may be one of the important causes of CBA sensitivity and of C57Bl/6 resistance to induction of this tumor. Since uterine sarcoma is a hormone-dependent tumor, and estrogen administration markedly stimulates its development [2], these data help explain the causes of interstrain differences in the induction of uterine sarcoma.

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Immunoglobulins of the Tumor-Bearing Host as Potential Regulators of the Tumor Recurrence Rate and Metastasis Development: a Study on the Model of Ehrlich Carcinoma

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On the model of Ehrlich carcinoma transplanted in mice it is shown that following the removal of the primary tumor the organism of operated mice produces factors enhancing the rate of relapses and metastasis. The tumor cells are shown to fix on their surface immunoglobulins capable of enhancing tumor development in the mice. The serum-derived tumor-enhancing immunoglobulins undergo active pinocytosis by the tumor cells. The fall in the level of these immunoglobulins is accompanied by a reduction of the rate of tumor growth.

Key Words: *Ehrlich carcinoma; recurrence; metastasis; immunoglobulins; mechanism of action*

Earlier we showed on the model of Ehrlich carcinoma that the surgical removal of the primary tumor fails to prolong the survival of mice. The operated animals died from relapses or metastases within the same time range as unoperated ones [1]. However, the unoperated mice did not develop metastases, their death being caused by the growth of the primary tumor. Thus, in this particular case the massive elimination of tumor cells resulted in no therapeutic effect. This conclusion is in conflict with the modern strategy of treatment of malignant diseases, which is based on the maximal removal of tumor cells from the organism. We speculated that the tumor-bearing organism can itself direct the growth of the solitary tumor cells left in the organism after the operation by boosting the development of relapses and metastases, thus canceling out the therapeutic effect of the operation. It follows from this assumption that the organism of a tumor-bearing individual produces certain substances capable of governing tumor growth. Although this assumption contradicts the prevailing view of autonomy of tumor development, it still represents a logical consequence of our earlier results.

In this report we have made an attempt to prove the existence of factors governing tumor growth in mice having undergone the surgical re-

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