Natural Killer Cell Activity and Tumor Susceptibility in Female Mice Treated Neonatally with Diethylstilbestrol

Terje Kalland and John-Gunnar Forsberg

Institute of Anatomy, University of Bergen, Bergen, Norway [T. K.], and Department of Anatomy, University of Lund, Biskopsgatan 7, S-223 62 Lund, Sweden [J-G. F.]

ABSTRACT

Female NMRI or AKR/J mice were given daily s.c. injections of 5 μg diethylstilbestrol (DES) in 0.025 ml olive oil, or of olive oil only, for the first 5 days after birth. At the age of 6 to 7 weeks, both DES-treated females and control females were killed, and the cytotoxic activity of the spleen cells against standard natural killer cell target YAC-1 cells as well as the natural killer cell-sensitive I-522 cells and relatively insensitive I-51 AKR lymphomas were tested. The cytotoxic activity against I-51 cells was similar for DES-treated and control females while the DES-treated females had only about one-half the cytotoxic activity to I-522 and YAC-1 cells as did controls. Control females eliminated radioactivity derived from 125I-labeled YAC-1 and I-522 target cells injected i.v. faster than did DES-treated females, while the results were similar for both animal groups when using I-51 cells. The cumulative death incidence was higher for DES-treated females than for control females after inoculation with low numbers of I-522 cells but similar for both groups when using I-51 cells. Finally, the incidence of females developing methylcholanthrene-induced sarcomas, using a single low-dose injection (10 or 20 μg), was higher among DES-injected animals than among controls. Taken together, the results indicate that female mice treated neonatally with DES have a functionally defective natural killer cell population, resulting in increased tumor susceptibility.

INTRODUCTION

Estrogen effects on the immune system are well documented (for review, see Ref. 43). Long-lasting treatment of adult animals results in involution of the thymus, induces lymphopenia, and impairs several aspects of the immune response. Antibody synthesis, delayed hypersensitivity response, T-cell cytotoxicity, mitogen responsiveness, and NK cell activity are all reduced after treatment with high doses of estrogens (21, 25, 40). The effects, however, are reversible, and the immune competence normalizes some time after cessation of treatment (1, 21).

In contrast, perinatal treatment of female mice with the nonsteroidal estrogen DES gives rise to persistent alterations in immunological functions, mainly through its interference with T-cell differentiation (17–21, 26, 50). Neonatal exposure to DES also leads to a persistent reduction in NK cell activity in several mouse strains (19). Later in life, female mice treated neonatally with estrogen develop adenocarcinomas or squamous cell carcinomas in the vagina and uterine cervix (6, 16, 46). These animals also have an increased incidence of mammary tumors and an increased mammary gland sensitivity to chemical carcinogens (14, 49).

Accumulating evidence indicates that NK cells may be of major importance in immunological surveillance against neoplasia and may also play a key role in the control of metastatic spreading of tumor cells (12, 13, 22, 23, 37). The NK-deficient mouse mutant beige has an increased susceptibility to transplanted tumors and also a marked increase in metastatic seeding from primary tumor cell inocula (22, 47).

Female mice exposed neonatally to DES and with a depressed NK cell activity may provide another model for testing the role of the NK cell in immune surveillance.

In this paper, we report studies aimed to analyze the potential activity of NK cells in vivo and in vitro of adult female mice exposed neonatally to DES to correlate this with the ability to resist development of primary MCA-induced sarcomas as well as growth of solid tumors from transplanted lymphoma cells with different sensitivity to NK cells.

MATERIALS AND METHODS

Animals. NMRI mice were from an outbred stock kept at our institute. Pregnant AKR/J mice were obtained from Olac, Ltd. (Oxfordshire, United Kingdom). Newborn females were separated into experimental and control groups, and treatment was begun within 24 hr after birth. Experimental females were given daily s.c. injections of 5 μg DES (Sigma Chemicals, London, United Kingdom) in 0.025 ml olive oil for the first 5 days after birth. Controls were given injections of the same amount of olive oil only. This treatment schedule has earlier been found to induce persistent changes in the immune system and to give rise to malignant changes in the uterine cervix of old animals (10).

Tumor Cells. The T-lymphoma cell lines I-522 and I-51, previously established in vitro from spontaneous thyromas of aged AKR/J females by Riesenfeld et al. (36), were obtained from Dr. Anders Ørn, Department of Immunology, University of Uppsala, Uppsala, Sweden. YAC-1 is a Moloney virus-induced lymphoma of A/Sn mouse origin. The cell lines were maintained in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and Fungizone (30 μg/ml) (complete medium). They were screened regularly for the presence of Mycoplasma.

In Vitro Cytotoxicity Assay. Spleen cells to be used as effector cells were prepared in physiological buffered saline from 6- to 7-week-old females. Mononuclear cells were separated on a Ficoll-Hypaque gradient by the method of Beyam (4). Target cells were labeled by incubation of 5 × 10⁶ cells in 0.5 ml complete medium with 100 μCi Na₂¹⁵⁶CrO₄ (specific activity 200 to 400 mCi/mg; The Radiochemical Centre, Amersham, United Kingdom) for 45 min at 37°C. The cells were washed once in 50 ml RPMI Medium 1640 and kept on ice for about 2 hr before they were washed twice more immediately before use in the assay. The cell number was adjusted to 2 × 10⁶/ml. One hundred
μl of the target cell suspension were added to various numbers of effector cells in 100 μl of medium and incubated in Greiner round-bottomed microtiter plates at 37° in a 5% CO₂ atmosphere for 5 hr. After centrifugation at 500 x g for 10 min, 100 μl of the supernatant were aspirated from each well, and the released ⁰⁹Cr was determined in a Searle 1185 gamma spectrophotometer. All experiments were performed with triplicates of effector:target cell ratios of 10:1, 50:1, and 100:1. Spontaneous release was determined by incubation of target cells in medium only and never exceeded 10%. Total radioactivity was determined in samples of the target cells. The percentage of cytotoxicity was determined by

\[
\text{Test cpm} - \text{spontaneous cpm} \times 100
\]

\[
\text{Total cpm} - \text{spontaneous cpm}
\]

In Vivo Cytotoxicity Assay. The in vivo cytotoxicity assay described by Riccardi et al. (34) was used. Extensive work by the same authors (35) has validated this assay as an in vivo estimation of NK activity. Fifty million tumor cells in 50 ml complete medium were incubated for 14 hr at 37° with 2.5 μCi ¹²⁵I-2'-deoxyuridine (5 Ci/mg; Radiochemical Centre) in the presence of 2'-deoxy-5-fluorouridine (0.2 μg/ml; Sigma) in order to prevent endogenous thymidine synthesis. After 3 washings with 50 ml medium, tumor cells were counted in the presence of trypan blue, and 10⁶ viable cells in 0.5 ml medium (corresponding to about 5 x 10⁶ cpm) were injected i.v. into the tail vein of 6- to 7-week-old female mice. At different times after tumor cell injection, groups of animals were killed, both lungs and spleen were dissected out, and radioactivity was measured in a Searle 1185 gamma spectrophotometer. Extraction with trichloroacetic acid and ethanol showed that more than 90% of the recovered radioactivity was cell bound. The results were expressed as recovered radioactivity in the organs at different times, given in percentage of injected radioactivity.

Tumor Cell Growth in Vivo. The AKR/J lymphomas I-522 and I-51 have been shown to have grossly similar growth patterns in low-NK-reactive AKR/J mice as measured by time-dependent increase in s.c. tumor size (48). The I-522 lymphoma displayed high and the I-51 lymphoma displayed low NK cell sensitivity in vitro. In the present study, 10² tumor cells in 0.1 ml RPMI Medium 1640 were inoculated into the necks of 6-week-old control mice or female AKR/J mice treated neonatally with DES. Tumor cell-inoculated mice were followed every day, and the day of animal death was recorded. The experiment was terminated 30 days after the last death of a tumor-bearing animal.

Induction of Sarcomas with MCA. At the age of 8 to 10 weeks, groups of DES-treated and control females (NMRI) were given a single s.c. injection of 10, 20, 50, 100, or 200 μg MCA (Sigma) in 0.05 ml tricaprolin (Sigma). The injection site was laterally on the right hind leg. At that time, the female was killed and the tumor was registered. Pieces of randomly chosen tumors from both DES-treated females and control females were fixed and prepared for histological study (hematoxylin-eosin staining of paraffin-embedded material). Care was taken to kill the females when all tumors had reached the same approximate size. All animals were followed during an observation period of 36 weeks. No autopsy diagnosis was tried on spontaneously dying females.

RESULTS

In Vitro Cytotoxicity Assay. The results of in vitro cytotoxicity assays of spleen cells from control females and females exposed neonatally to DES are shown in Table 1.

The earlier reported decrease of NK cell activity in mice exposed neonatally to DES (19) was confirmed using the NK cell-sensitive tumor cell line YAC-1. A pronounced difference in sensitivity to lysis by spleen cells from control animals was observed for the 2 AKR/J lymphomas tested, I-522 being sensitive while I-51 was almost totally resistant. No significant difference in cytotoxicity towards the I-51 cell line was detected when spleen cells from control animals and animals exposed neonatally to DES were compared. On the other hand, neonatal DES treatment resulted in a statistically significant (Student's t test; 0.05 > p > 0.01) and about 50% reduction in cytotoxicity to I-522 cells. This reduction was about the same when YAC-1 cells were used.

In Vivo Elimination of Radiolabeled Tumor Cells. The radioactivity was determined in the spleen and lungs at different times after i.v. injection of ¹²⁵I-2'-deoxyuridine-labeled tumor cells in an allogeneic (YAC-1 cells into female NMRI mice) as well as in a syngeneic (I-522 or I-51 cells into female AKR/J mice) system. The decrease of radioactivity, reflecting clearance of labeled YAC-1 cells from spleen and lungs of control mice and female NMRI mice treated neonatally with DES, is shown in Chart 1. The radioactivity decreased more rapidly from both organs in control females compared with DES-treated females. The difference was most evident for the lungs with a relatively high remaining radioactivity in DES females 4 hr after injection of the tumor cells.

| Table 1: Effect of neonatal DES treatment of female AKR/J mice on NK activity against lymphoma cell lines in vitro |
|-----------------|-----------------|-----------------|
|                  | I-51            | I-522           | YAC-1           |
| Control          | 0.9 ± 0.1       | 20.1 ± 3.1      | 24.8 ± 2.7      |
| DES-treated      | 1.3 ± 0.3       | 9.7 ± 1.8       | 11.3 ± 1.5      |

*Mean ± S.E. in 5 females assayed individually in every group.
the lungs (Chart 2).

The difference in YAC-1 and I-522 cell clearance between DES and control females is striking for both spleen and lungs. Four hr after inoculation, control females had cleared approximately 4 to 5 times as much radioactivity, representing YAC-1 and I-522 cells, as had DES-treated females. For the spleen, approximately twice as much radioactivity cleared from control females as from DES-treated females.

When the relatively NK-resistant cell line I-51 was injected, no difference in the ability to eliminate the tumor cells was seen between the 2 animal groups. A substantial amount of radioactivity was found in the lungs 4 hr after tumor cell injection (Chart 2).

**In Vivo Tumor Growth.** The cumulative death incidence of control females and females treated neonatally with DES after transplantation of I-522 or I-51 lymphoma cells into AKR/J mice is shown in Chart 3. All the females had large tumors at death.

When the relatively NK-resistant tumor I-51 was used, a high percentage of the animals developed tumors. No difference in tumor-associated death incidence was revealed when females treated neonatally with DES were compared with control females. At the end of the experiment, about 90% of the females in both groups had died. The first deaths occurred about 1 month after transplantation.

Inoculation of cells from the NK-sensitive cell line I-522 resulted in a significant higher percentage of deaths among DES-treated females than in controls. Moreover, the first deaths occurred about 1 month earlier in DES-treated females than in controls. Thus, females treated neonatally with DES were much more susceptible to tumor cell inoculation. At the end of the observation period, about 75% of the DES-treated females had died as opposed to about 30% of the controls.

**Induction of Primary Tumors with MCA.** The histological picture of the sectioned tumor material was that of typical sarcomas, without any obvious differences related to the type of host (DES-treated females or controls) of the tumors.

The yield of local sarcomas and cumulative death incidence as a function of time after injection of MCA at different dose levels is shown in Chart 4. A common trend for all dose groups is that the tumors appeared slightly earlier among DES-treated females than among controls, and with a possible tendency to later appearance in the 10- and 20-μg groups compared with higher dose levels. Further, after injection of 50 μg or lower doses, the yield of sarcomas was consistently higher among DES-treated females than among controls, and with a possible tendency to later appearance in the 10- and 20-μg groups compared with higher dose levels. Further, after injection of 50 μg or lower doses, the yield of sarcomas was consistently higher among DES-treated females than among controls, and with a possible tendency to later appearance in the 10- and 20-μg groups compared with higher dose levels. Further, after injection of 50 μg or lower doses, the yield of sarcomas was consistently higher among DES-treated females than among controls, and with a possible tendency to later appearance in the 10- and 20-μg groups compared with higher dose levels. Further, after injection of 50 μg or lower doses, the yield of sarcomas was consistently higher among DES-treated females than among controls, and with a possible tendency to later appearance in the 10- and 20-μg groups compared with higher dose levels. Further, after injection of 50 μg or lower doses, the yield of sarcomas was consistently higher among DES-treated females than among controls, and with a possible tendency to later appearance in the 10- and 20-μg groups compared with higher dose levels. Further, after injection of 50 μg or lower doses, the yield of sarcomas was consistently higher among DES-treated females than among controls, and with a possible tendency to later appearance in the 10- and 20-μg groups compared with higher dose levels.
for DES-treated females the yield was relatively constant at all doses used. These results are more evident from Chart 5 where the total incidence of tumors at the end of the observation period is plotted for the different dose levels. For the controls, there was a probable significant regression of total tumor yield at 36 weeks on the MCA dose ($F = 34.11$ for 1 and 3 d.f.; $0.05 > p > 0.01$). For DES-treated females, there was no such regression or dose-response relationship. These results should be compared with the mortality after different MCA doses (Chart 5). For control females, the mortality was relatively constant for all MCA doses used, while there is a significant regression of mortality on MCA dose for DES-treated females ($F = 37.73$ for 1 and 3 d.f.; $0.01 > p > 0.001$).

Single doses of MCA higher than 50 μg have a long-lasting immunosuppressive effect (see "Discussion"). Such an immunosuppressive effect could be thought to influence both tumor yield and mortality, except when using a 10- or 20-μg dose. Thus, a special analysis was made of the results obtained from these 2 latter dose groups. An adjusted $\chi^2$ analysis did
itself has a long-lasting immunosuppressive effect, demonstrable for doses higher than 50 μg (44, 45). The effect of MCA on NK activity has to our knowledge not been tested while 2 other carcinogens, urethan and 7,12-dimethylbenz(a)anthracene, have been shown to depress NK activity (7). For the 2 lowest doses used, 10 and 20 μg, it could be demonstrated that the DES-injected females gave a significantly higher yield of sarcomas than did controls. The time from MCA injection to appearance of first tumor tended to be somewhat longer with the same doses compared with the higher ones. In the beige mouse mutation, known to abrogate NK activity, no effect was seen on induction of primary sarcomas with 100 μg MCA, consistent with our findings with high doses of MCA in DES-induced NK deficiency (39). Because the dose of MCA is related to the immunogenicity of the tumors (2, 32, 33), tumors induced by low doses of MCA may escape a weak immune response in the DES-treated females to a higher degree than that of the normal response in control females. A weak immune response has been described to have a more stimulating effect on tumorigenesis than either a minimal or maximal capacity (31, 33). Thus, our results could also be in line with some type of tumor stimulation in DES-treated females.

Studies on death rate do not indicate that DES-treated females given injections of 10 to 50 μg MCA are healthier than the controls; thus, the difference in tumor incidence is not explained on a simple “nonimmunological” basis. Increasing the doses of MCA to 100 or 200 μg did not influence the death rate of the controls, but there was a pronounced increase for the DES-treated females. With these high doses of MCA, the tumor yield did not increase in DES-treated females but did increase in the controls. DES females given injections of the higher doses of MCA apparently die too early to develop tumors. No autopsy of the spontaneously dying females was undertaken; therefore, the specific cause of the deaths is unknown.

Based on the results from the studies on NK cell activity presented in this paper, as well as on the general discussion on NK cell effects, it seems pertinent to associate the increased yield of MCA-induced sarcomas in females treated neonatally with DES to their decreased NK cell activity.

Now, turning to the initial question of the importance of different factors (cellular, hormonal, immunological) for the development of mammary and genital tumors in female mice given neonatal injections of estrogen, it seems reasonable to conclude that a disturbed immune function is relevant. However, because the quite dominant tumor localization in such females is in estrogen target tissues, the primary carcinogenic hit may be exerted in the neonatal period at the target cellular level (10). Hormonal and immunological factors may later interact for a final expression of malignancy. Depending on target tissue, the relative importance of hormonal and immune factors may vary. Thus, hormonal factors may play a relatively greater role for the development of mammary tumors than immune alterations might (27, 42), while the reverse may be true for cervicovaginal tumors.

REFERENCES

Natural Killer Cell Activity and Tumor Susceptibility in Female Mice Treated Neonatally with Diethylstilbestrol

Terje Kalland and John-Gunnar Forsberg