0022-1767/78/1216-2193$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 121, No. 6, December 1978
Printed in U.S.A.

β-ESTRADIOL REDUCES NATURAL KILLER CELLS IN MICE[1]

WILLIAM E. SEAMAN, MARCIA A. BLACKMAN, THOMAS D. GINDHART, JIRAYR R. ROUBINIAN, JOHN M. LOEB, AND NORMAN TALAL

From the Immunology/Rheumatology Division and the Department of Pathology, Ft. Miley Veterans Hospital and the University of California, San Francisco, California

β-estradiol was administered to mice continuously by diffusion from a silastic tube that was implanted subcutaneously at 4 weeks of age. Four to 6 weeks of estrogen administration caused a substantial reduction in natural killer cell activity in the spleens from mice of either sex. Androgen (5α-dihydrotestosterone) did not. Castration of male or female mice did not affect natural killing and did not alter the effect of β-estradiol. Estradiol did not affect natural killing *in vitro* and the loss of natural killing was not due to a soluble or a cellular suppressor of natural killing. The effects of estradiol were not dependent on the thymus, since estradiol reduced natural killing in mice that had been neonatally thymectomized. After removal of the estrogen implant, natural killing recovered over a period of 8 weeks. The loss of natural killing may reflect a loss of bone marrow secondary to estrogen-induced osteosclerosis.

Natural killer (NK) cells are lymphocytes that rapidly kill certain tumors and transformed cell lines *in vitro* despite a lack of known sensitization to the target (1, 2). NK cells lack the characteristics of mature T cells or B cells (2). In mice, they are most prominent in the spleen and the peripheral blood. They are less evident in lymph nodes and bone marrow, and are absent from the thymus (2, 3). NK activity first appears in the spleen when the mouse is about 20 days old. It then rises rapidly, but declines after 3 to 4 months. NK activity does not differ between male and female mice (3), but the effect of sex hormones on natural killing has not otherwise been investigated. We demonstrate here that pharmacologic amounts of estrogen, given chronically, will substantially lower NK activity in the mouse.

Our laboratory is interested in the role of NK cells and the effects of sex hormones in the NZB/NZW F_1 mouse, which develops an autoimmune disease resembling systemic lupus erythematosus (4). We have therefore carried out many of our studies in this strain, which has high levels of natural killing. Similar results, however, are demonstrated in normal mouse strains.

Received for publication July 12, 1978.
Accepted for publication September 1, 1978.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by the Department of Health, State of California, Grant No. 76-57090, by funds from the Academic Senate, University of California, San Francisco, and by the Medical Research Service at the Veterans Administration.

MATERIALS AND METHODS

Mice. NZB/NZW mice were raised in our vivarium by using NZB/J females (Jackson Laboratories, Bar Harbor, Maine) and NZW/NIH males. C57BL/6, C57BL/ 10Sn, DBA/2, B10.D2ns, and BALB/c mice were purchased from Jackson Laboratories.

Medium. Cell preparation and *in vitro* assays for natural killing were carried out in RPMI 1640 with 25 mM HEPES buffer (GIBCO, Grand Island, N. Y.) plus 5% fetal calf serum (FCS) (GIBCO, lot No. C979224). For the NK assay, FCS was increased to 20% and the medium supplemented with 2mM glutamine, 50 μg/ml penicillin G, and 100 μg/ml streptomycin.

Cell preparation. The *target* for natural killing was YAC-1, a Moloney virus-induced lymphoma from A/J mice (kindly supplied by Dr. George Klein) that we carry *in vitro* in RPMI 1640 plus 10% FCS. Target cells were labeled by incubation with ^{51}Cr (New England Nuclear, Boston, Mass.) for 1 hr, washed three times, and resuspended to 4×10^5 live cells/ml. *Effector cells* were obtained from mice that had been killed by cervical dislocation. Cells were collected from the spleen, lymph node, or thymus by mincing the organ and teasing cells through 40-mesh steel screen. Red cells were lysed by incubation in 0.155 M ammonium chloride for 5 min on ice, using bicarbonate buffer, pH 7.4, supplemented with 10% FCS. (We find that incubation in ammonium chloride either does not change or slightly increases natural killing. An increase is generally seen when testing spleens with many red cells, e.g., from an old NZB mouse.)

Assay for natural killing. The assay was carried out in round-bottom microtiter plates (Linbro Chemicals, New Haven, Conn). Effector cells in 150λ were added to 50λ of target cells with the effectors at varying concentrations to give effector: target cell ratios of 80:1, 40:1, 20:1, and 10:1. Triplicate cultures were performed for each concentration. The cultures were incubated at 37°C in 5% CO_2 for 5 hr, then centrifuged at 500 × G for 10 min. Supernatant (100λ) from each well was aspirated and diluted with 1.0 ml water. Released ^{51}Cr in the supernatant was determined in a Packard gamma scintillation spectrometer. Maximum release was determined by incubation of labeled target cells in 15% saponin, which regularly releases 95 to 100% of radioactivity. Spontaneous release was determined by using unlabeled target cells as effectors and was always less than 20% of maximum release. Percent cytotoxicity was determined by:

$$\frac{^{CPM}\text{effectors} - {}^{CPM}\text{spontaneous release}}{^{CPM}\text{maximum release} - {}^{CPM}\text{spontaneous release}} \times 100.$$

Each cytotoxicity curve represents the mean of 3 to 6 mice assayed individually. In agreement with previous workers, we

find that cytotoxicity in this assay is increased or unchanged if effectors are a) pretreated with anti-Thy 1.2 plus complement (C), b) depleted of cells adherent to plastic, or c) depleted of B lymphocytes on nylon wool columns (Ig^+ cells reduced from 50 to 2% as detected by fluorescence).

A few of the assays were carried out in Petri dishes instead of microtiter plates by using the method of Canty and Wunderlich (5), with 5×10^4 target cells in a volume of 1.0 ml and with effector:target cell ratios as in the microtiter assay. The results with this assay are similar to those obtained with the microtiter assay.

We have chosen to present the entire killing curve for each experiment rather than calculate a killing index or lytic units (6). This is because a) killing by estrogen-treated spleen cells was often very low—less than 10% even at an effector:target ratio of 160:1, and b) the killing curve for untreated mice was not parallel to the curve for estrogen-treated mice. Estrogens, however, induce splenomegaly, so it is important to consider whether changes in the cytotoxicity curve reflect changes in natural killing *per spleen*. The splenomegaly due to estrogens is due primarily to an increase in red cells: the mean number of splenic white cells recovered from mice that had received estrogens for 6 weeks was 20% *less* than the number recovered from sham-treated mice (average for all experiments). Thus, our results may actually underestimate the reduction in natural killing caused by estrogens in the spleen as a whole.

Treatment with sex hormones. The chronic administration of sex hormones was achieved by the subcutaneous implantation of Silastic tubing (Dow-Corning, Midland, Mich.) containing hormone, as described previously (7). The tubing (inner diameter 0.062 inches, outer diameter 0.125 inches) was cut into 2.0-cm segments and filled with 17 β-estradiol (Sigma Chemical, St. Louis, Mo.), with 5α-dihydrotestosterone (Sigma) or with nothing (sham implants). When lightly packed, each implant contained about 15 mg of either hormone. The ends of the tubing were sealed with Silastic medical adhesive (Dow-Corning) and dried for 48 hr at 37°C. For the insertion of implants, mice were anesthetized by using i.p. pentobarbital. A 5 to 10 mm skin incision was made over the dorsum, and the subcutaneous tissues were lysed with a blunt probe. The implant was inserted into the subcutaneous space and the wound was sealed with metal clips. All mice were given implants at 4 weeks (24 to 32 days). For removal of implants, a new incision was made.

Castration. Mice were castrated at 4 weeks by using pentobarbital anesthesia. Mice receiving implants had the two procedures at the same time. Testes were delivered through a scrotal incision and excised by using electrocautery. The incision was closed with 6-0 silk. Ovaries were removed through bilateral 5 to 10 mm incisions over the dorsum, which were closed with metal clips. For sham operations, the gonads were identified and the wound then closed.

Neonatal thymectomy. Thymectomy was performed within 24 hr of birth, as described previously (8). By using hypothermic anesthesia, the sternum was incised from manubrium to the sixth rib, and the thymus was gently mobilized by using light suction. The skin was closed with 6-0 silk.

RESULTS

Effects of estrogen (β-estradiol) or androgen (5α-dihydrotestosterone) in castrated mice. NZB/NZW and B10.D2ns mice of both sexes were castrated at 4 weeks and were given implants containing androgen or estrogen. Six weeks later, mice from either sex that had received estrogen were found to have a marked deficiency of natural killing by spleen cells, regardless of whether the recipients were male or female (Fig. 1). Androgen reduced natural killing only slightly or not at all (Fig. 1). Natural killing by lymph node cells was similarly reduced by estrogens, whereas natural killing by thymocytes was barely detectable in either sham- or in hormone-treated mice. For reasons that will be discussed, estrogen-treated mice had insufficient bone marrow cells to examine their NK activity.

Effect of castration alone and the effect of estrogen in uncastrated mice. Castration alone had no effect on splenic natural killing over the same time period in NZB/NZW mice. Moreover, estrogen therapy effectively depleted natural killing in mice that had not been castrated (Fig. 2). The effect of estrogen on natural killing was then tested in noncastrated C57BL/10, B10.D2ns, DBA/2, and BALB/c mice. In all strains and in either sex, 6 weeks of estrogen led to a significant reduction in natural killing by spleen cells (Fig. 3).

Time course of the loss of natural killing. As shown in Figure 4, treatment of male mice with β-estradiol for 2 weeks had little effect on natural killing, but natural killing was substantially reduced by 4 to 6 weeks. Similar results were obtained in female mice.

Effect of removing the estrogen implant after 6 weeks. Male NZB/NZW mice were treated with estrogens for 6 weeks and then the implant was removed. As shown in Figure 5, there was no recovery of natural killing 2 weeks after removal of the implant. After 4 weeks, there was partial recovery; after 8 weeks natural killing was fully restored.

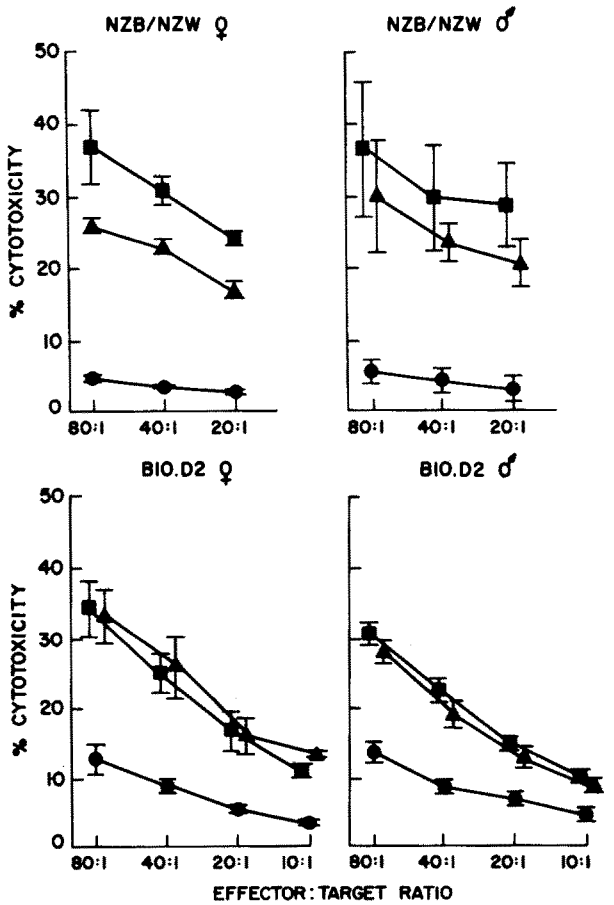

Figure 1. Natural killing by spleen cells from castrated mice treated with β-estradiol (●—●), 5α-dihydrotestosterone (▲—▲), or sham implant (■—■).

Figure 5 also demonstrates that continuation of estrogen therapy beyond 6 weeks resulted in continued suppression of natural killing. After 14 weeks of therapy, there was virtually no NK activity in the spleens from estrogen-treated mice.

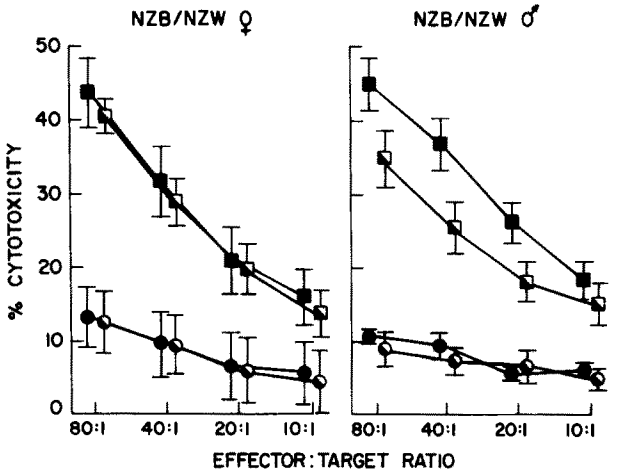

Figure 2. Effect of 6 weeks of β-estradiol on normal *vs* castrated mice. Mice were treated with β-estradiol alone (●—●), β-estradiol plus castration (◔—◔), sham implant (■—■), or sham implant plus castration (◪—◪).

Lack of cellular or humoral suppression of natural killing in estrogen-treated mice. Natural killing by spleen cells from sham-treated mice was not reduced by the addition of spleen cells from estrogen-treated mice over a wide range of cell dilutions (Fig. 6). The results shown are for NZB/NZW mice. Similar results were found with C57BL/6 mice. These mixing experiments also demonstrate that the addition of normal cells to spleen cells from estrogen-treated mice did not reconstitute killing by the estrogen-treated cells.

Natural killing by normal spleen cells was also not suppressed by humoral factors from estrogen-treated mice: *In vitro* assays of natural killing in which mouse serum (1%, 2%, 4%, or 8%) was substituted for FCS showed no difference between serum from sham-treated mice and serum from estrogen-treated mice (data not shown). Also, the direct addition of β-estradiol to the *in vitro* assay for natural killing in concentrations from 10^2 to 10^4 nanograms/ml did not suppress killing.

Effect of estrogen in neonatally thymectomized mice. NK cells are present in nude (athymic) mice, indicating that they develop without thymic influence (2). However, because estrogens are thymolytic in rodents (9), we examined the effect of β-estradiol in neonatally thymectomized mice in order to examine the hypothesis that estrogen might alter natural killing

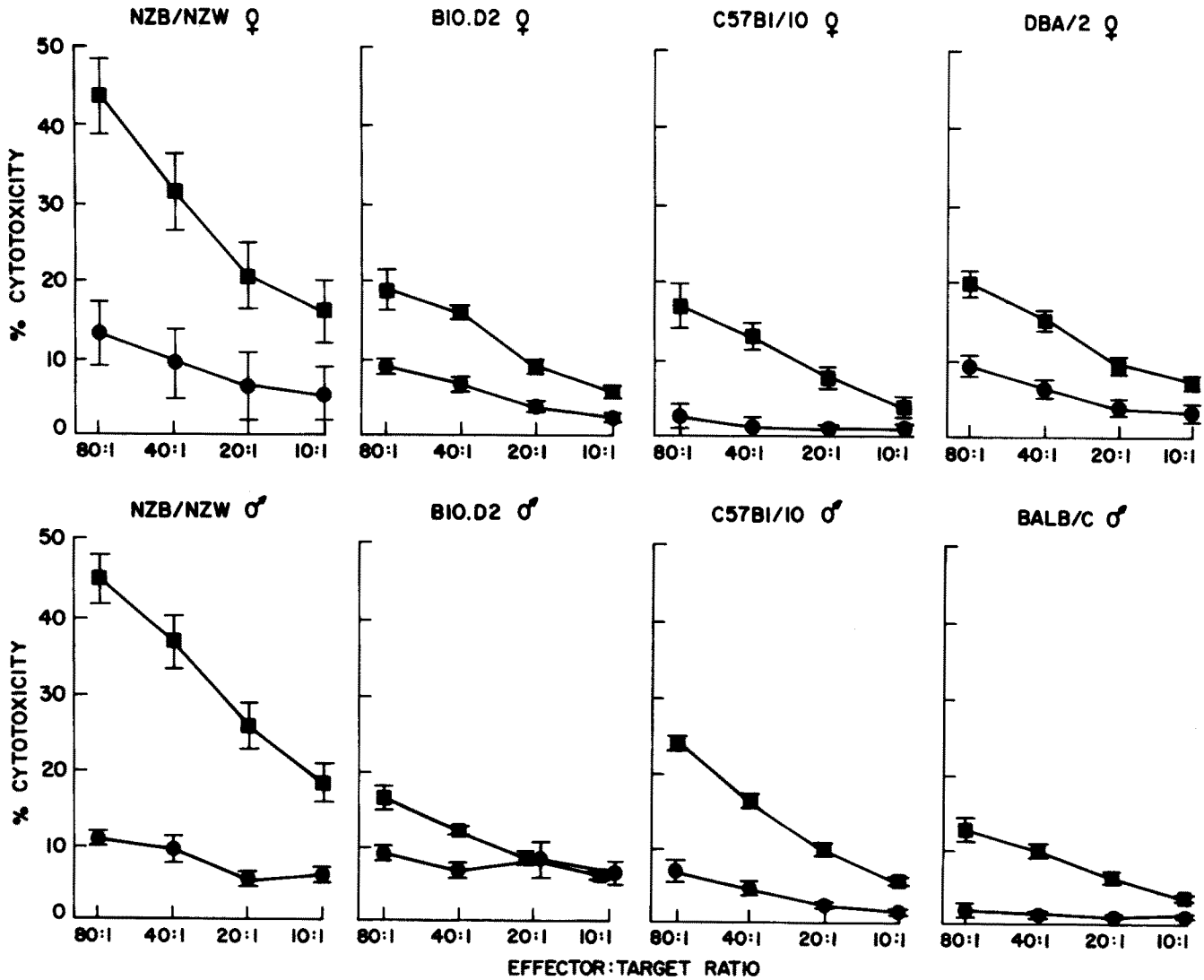

Figure 3. Effect of β-estradiol in (uncastrated) mice. Mice were treated for 6 weeks with β-estradiol (●—●) or sham implant (■—■). *Upper row,* female mice; *lower row,* male mice.

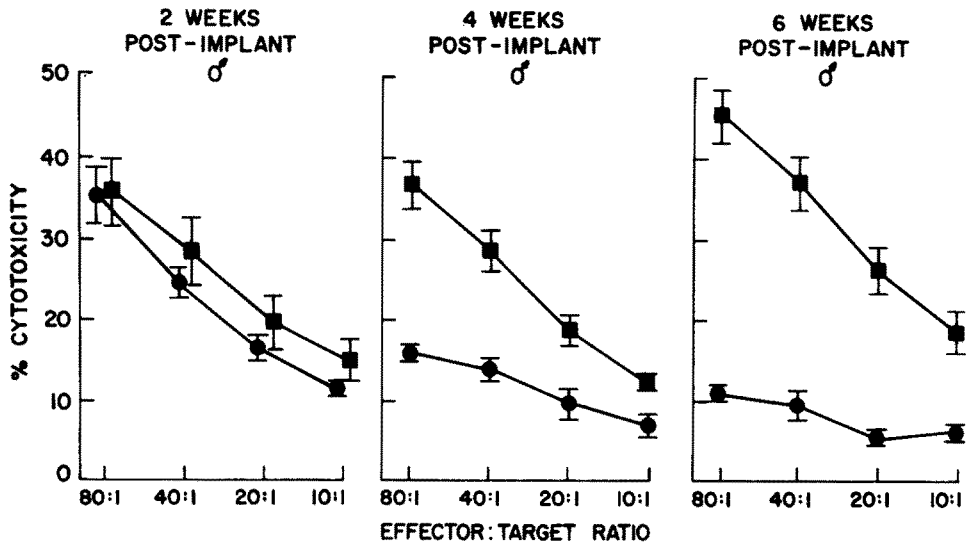

Figure 4. Time course of the effect of β-estradiol on natural killing in NZB/NZW male mice. Mice were given either β-estradiol (●—●), or sham implant (■—■), at 4 weeks.

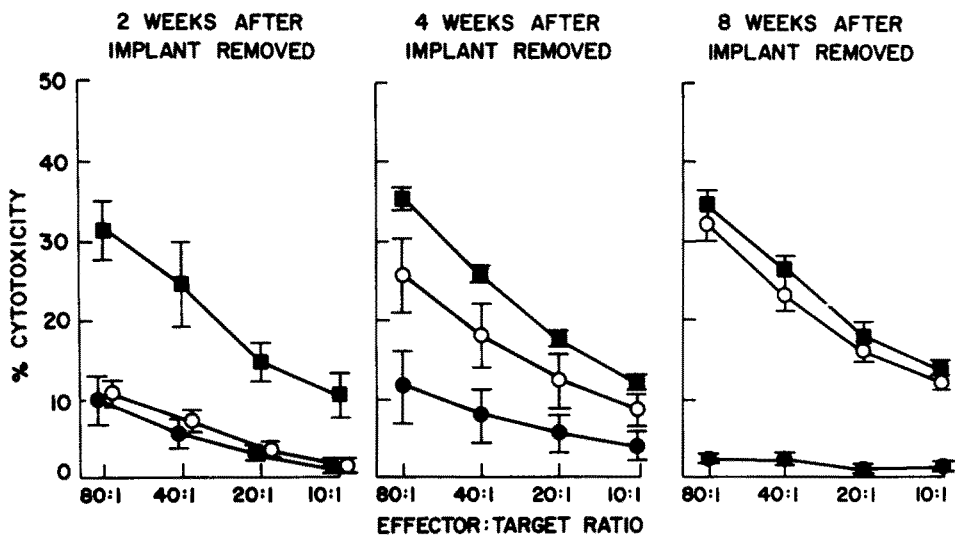

Figure 5. Recovery of natural killing after β-estradiol treatment. Mice received either estradiol or sham implant for 6 weeks. In half the mice the implant was then removed. The figure shows killing by animals that continued to receive β-estradiol (●—●) or in which the β-estradiol implant was removed (○—○) as well as animals that had a sham implant removed after 6 weeks (■—■). The curve for mice in which a sham implant was left was the same as, or only slightly lower than, the curve for animals in which the implant was removed.

by creating an imbalance among thymocyte subpopulations. As shown in Figure 7, neonatally thymectomized mice were as susceptible to the effects of estrogens as sham-operated mice.

DISCUSSION

We have demonstrated that the chronic administration of 17 β-estradiol to mice reduces natural killing over a period of several weeks. This occurs in both male and female mice, whether castrated or not. In untreated or in castrated mice there is no difference between males and females with regard to natural killing. This implies that the effects of estrogen in these experiments are pharmacologic rather than physiologic. The mice in these experiments were given estrogen beginning at four weeks, but we have observed a similar reduction of natural killing in mice treated at 12 weeks (unpublished).

There are at least three possible mechanisms for the reduction of natural killing by estrogens: 1) a reduction in the number of natural killer cells, 2) active suppression of the effectiveness of natural killer cells, or 3) a reduction in the number (or effectiveness) of accessory cells that are required for natural killing. Our experiments favor the first of these three possibilities, a reduction in the number of natural killer cells. Suppression of natural killing could not be demonstrated by using either cells or serum from estrogen-treated mice. Similarly the loss, or the inhibition, of an accessory cell was not seen in experiments in which cells from sham-treated mice were mixed with cells from estrogen-treated mice. The normal cells provided only the amount of cytotoxicity expected for their own NK cells; they did not promote killing by the estrogen-treated cells.

Estrogens are not directly toxic to mature NK cells, or at least not rapidly so. The addition of estrogens to the *in vitro* assay for natural killing did not reduce cytotoxicity, and a reduction in natural killing *in vivo* required more than 2 weeks of estrogen therapy. These findings suggest that estrogens affect the generation of NK cells.

A reduction in the generation of NK cells might be due either to a reduction in the number of NK precursor cells or to an alteration in the host that retards the generation of NK cells from precursor cells. With regard to the later possibility, we considered two prominent effects of estrogen on the host involving, respectively, the thymus and bone. First, estrogens are thymolytic (9). This thymolytic effect does not appear to account for the loss of natural killing since we found that estrogens reduce natural killing in neonatally thymectomized mice. Second, estrogens induce osteosclerosis, with replacement of bone marrow by osteoid bone (10). This effect is seen with the amounts of estrogen used in these experiments. We are finding extensive osteosclerosis with loss of bone marrow in mice from all strains that have received estrogens for 4 weeks or more. This effect of estrogens may contribute to their effect on NK cells since NK cells derive from the marrow (11) and are

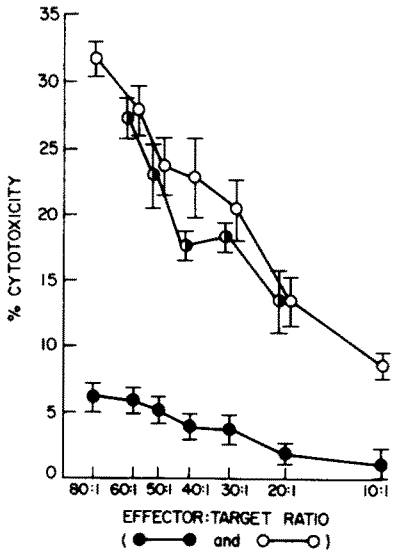

Figure 6. Natural killing by a mixture of spleen cells from NZB/NZW female mice that were treated for 6 weeks with β-estradiol or with sham implant. Effector to target cell ratios are shown for estrogen-treated cells (●—●) and for sham-treated cells (○—○). For the mixture of estrogen-treated and sham-treated cells (◑—◑), the sham-treated cells were again in the effector to target cell ratio shown on the abscissa. Estrogen-treated cells were added in sufficient numbers to give a constant effector to target cell ratio of 80:1. For example, when sham-treated cells are at 20:1, estrogen-treated cells are at 60:1.

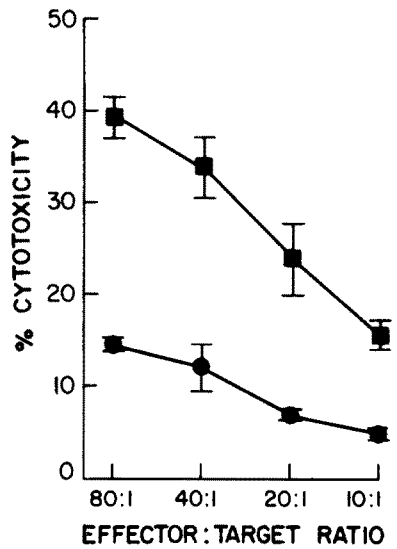

Figure 7. Natural killing by spleen cells from NZB/NZW mice that were neonatally thymectomized. Starting at 4 weeks, the mice were treated for 6 weeks with either β-estradiol (●—●) or sham implant (■—■).

dependent on the marrow for their continued generation (12). We are now studying in detail the extent of osteosclerosis in mice treated by our protocol and are examining the effect of estrogens on another marrow-dependent function, genetic resistance to bone marrow transplantation (13).

The effect of estrogens on cellular and humoral immunity has been examined by a number of investigators with varying results. To some degree, these variations may reflect differences in the estrogen chosen, dosage, route of administration, species, age, and experimental protocol for examining immunity (7, 14–17). In general, however, physiologic levels of estrogens appear to enhance humoral immunity in the mouse (17). Similarly, physiologic levels of estrogens appear to accelerate autoimmunity in that Nafoxidine, an estrogen inhibitor, delays autoimmunity in the NZB/NZW mouse (18). Pharmacologic levels of β-estradiol, given by the same protocol used in our experiments, accelerate disease (7). Whether the latter effect is related to the effect of β-estradiol on natural killing is unknown. Natural killer cells are thought to be important in host defense against malignancy (19). A reduction in natural killer cells may therefore contribute to the increased incidence of lymphoreticular malignancy in estrogen-treated mice (20, 21).

The loss of natural killer cells after treatment with estrogens provides a new model for studying the regulation of these cells. In particular, the delay in recovery of natural killer cells after cessation of estrogen treatment allows the study of mice in whom NK cells have been depleted but who are free from the agent that destroyed them.

REFERENCES

1. Herberman, R. B., M. E. Nunn, H. T. Holden, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int. J. Cancer 16:230.
2. Kiessling, R., E. Klein, N. Pross, and H. Wigzell. 1975. Natural killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur. J. Immunol. 5:117.
3. Kiessling, R., E. Klein, and H. Wigzell. 1975. Natural killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur. J. Immunol. 5:112.
4. Talal, N., and A. D. Steinberg. 1974. The pathogenesis of autoimmunity in New Zealand Black mice. Curr. Top. Microbiol. Immunol. 64:79.
5. Canty, T. G., and J. R. Wunderlich. 1970. Quantitative *in vitro* assay of cytotoxic cellular immunity. J. Natl. Cancer Inst. 45:761.
6. Herberman, R. B., S. Bartram, J. S. Haskill, M. Nunn, H. T. Holden, and W. M. West. 1977. Fc receptors on mouse effector cells mediating natural cytotoxicity against tumor cells. J. Immunol. 119:322.
7. Roubinian, J. R., N. Talal, J. S. Greenspan, J. R. Goodman, and P. K. Siiteri. 1978. Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F_1 mice. J. Exp. Med. 147:1568.
8. Roubinian, J. R., R. Papoian, and N. Talal. 1975. Effects of neonatal thymectomy and splenectomy on survival and regulation of autoantibody transformation in NZB/NZW F_1 mice. J. Immunol. 118:1524.
9. Dougherty, T. F. 1952. Effect of hormones on lymphatic tissue. Physiol. Rev. 32:379.
10. Urist, M. R., A. M. Budy, and F. C. McLean. 1950. Endosteal bone formation in estrogen-treated mice. J. Bone Joint Surg. 32-A:143.
11. Haller, O., R. Kiessling, A. Örn, and H. Wigzell. 1977. Generation of natural killer cells: an autonomous function of the bone marrow. J. Exp. Med. 145:1411.
12. Haller, O., and Wigzell, H. 1977. Suppression of natural killer cell activity with radioactive strontium: effector cells are marrow-dependent. J. Immunol. 118:1503.
13. Bennett, M. 1973. Prevention of marrow allograft rejection with radioactive strontium: evidence for marrow-dependent effector cells. J. Immunol. 110:510.
14. von Haam, E., and I. Rosenfeld. 1942. The effect of estrone on antibody production. J. Immunol. 43:109.
15. Kappas, A., and R. Palmer. 1963. Selected aspects of steroid pharmacology. Pharmacol. Rev. 15:123.
16. Hulka, J. F., K. Mohr, and M. W. Lieberman. 1965. Effect of synthetic progestational agents on allograft rejection and circulating antibody production. Endocrinology 77:897.
17. Eidinger, D., and T. J. Garrett. 1972. Studies of the regulatory

effects of the sex hormones on antibody formation and stem cell differentiation. J. Exp. Med. 136:1098.

18. Duvic, M., A. D. Steinberg, and L. W. Klassen. 1978. Effect of the anti-estrogen, nafoxidine, on NZB/W autoimmune disease. Arthritis Rheum. 21:414.
19. Haller, O., M. Hansson, R. Kiessling, and H. Wigzell. 1977. Role of non-conventional natural killer cells in resistance against syngeneic tumour cells *in vivo*. Nature 270:609.
20. Gardner, W. U., T. F. Dougherty, and W. L. Williams. 1944. Lymphoid tumors in mice receiving steroid hormones. Cancer Res. 4:73.
21. Kirschbaum A., J. R. Shapiro, and H. W. Mixer. 1953. Synergistic action of leukemogenic agents. Cancer Res. 13:262.