EFFECT OF ESTROGEN ON NATURAL KILLER CELLS

WILLIAM E. SEAMAN and THOMAS D. GINDHART

Treatment of mice with sustained high levels of β-estradiol leads to a reduction in natural killer cell activity and genetic resistance to bone marrow transplantation. The loss of natural killing does not seem to result from either humoral or immune suppression. Natural killer cells are thought to depend on the bone marrow, and it is notable that estrogens reduce natural killing at approximately the same time that they produce a loss of marrow due to osteoproliferation. Similarly, mice with congenital osteopetrosis are deficient in natural killing. However, changes in natural killing during and after treatment with estrogen do not correspond directly to changes in marrow volume. Estrogens are known to exacerbate spontaneous autoimmunity in NZB/NZW mice. The relationship between this effect and the effect of estrogen on natural killing is not clear. When natural killing is lowered in NZB/NZW mice by the in vivo administration of ^{89}Sr, autoimmunity is reduced.

We have been studying the effects of β-estradiol on natural killer cells in the mouse (1,2). Such cells are stimulated by viral infection (3), and though they do not appear to recognize viral antigens, they may nonetheless be important in defense against viral infection (4).

Natural killing is the ability of certain cells, which appear to be small lymphocytes, to rapidly lyse certain tumors and transformed cell lines in vitro without prior sensitization to the target (5,6). Certain T cell lymphomas are among the best targets for natural killing, and recent evidence suggests that natural killer cells can destroy certain normal T cells as well as malignant cells (7). The cells that mediate natural killing, natural killer cells (NK cells), are most abundant in the spleen and peripheral blood and are found to a lesser extent in the lymph nodes and bone marrow. They are absent from the thymus and nonlymphoid organs. NK cells are not mature T or B cells, but they may be related to T cells in that some workers have found low levels of θ antigen on the surface of mouse NK cells (8).

In mice, natural killing is absent for the first 3 weeks of life. This activity then increases rapidly and is sustained for 1–2 months, after which it gradually declines. Levels of natural killing differ between various inbred mouse strains. The NZB/NZW (B/W) F_1 mouse has particularly high levels of natural killing. Because of this and our interest in the autoimmune disease that develops in these mice, we have studied the effects of estradiol on natural killing in these mice as well as in normal mice. As a target for natural killing, YAC–1, a Moloney virus–induced T cell lymphoma from A/J mice was used. Target cells were labeled with ^{51}chromium and incubated in the presence of effector cells at varying ratios for 5 hours, after which the release of chromium into the supernatant was measured. Unless otherwise stated, we used spleen cells as effectors.

To study the effect of estrogen on natural killing,

From the Immunology/Rheumatology Division, Fort Miley Veterans Administration Hospital and the Departments of Medicine, Oral Medicine, and Pathology, University of California, San Francisco.

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 of the Veterans Administration.

William E. Seaman, MD: Staff Physician, Veterans Administration Medical Center, San Francisco, and Assistant Professor of Medicine; Thomas D. Gindhart, MD: Resident in Pathology, University of California, San Francisco.

Address reprints to William E. Seaman, MD, VAMC 151-T, 4150 Clement Street, San Francisco, CA 94121.

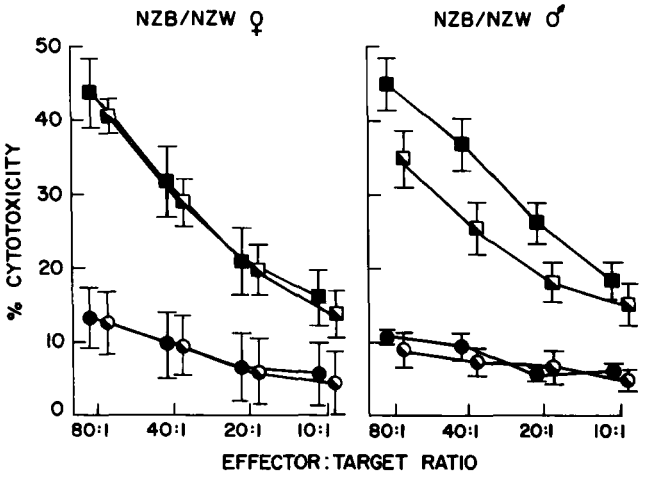

Figure 1. Effect of 6 weeks of 17β-estradiol on NZB/NZW female or male mice. Mice were treated with β-estradiol alone (solid circles), with β-estradiol plus castration (half-shaded circles), with sham implant alone (solid squares), or with sham implant plus castration (half-shaded squares). (From Seaman WE et al. J Immunol 121:2193–2198, 1978. Copyright 1978, The Williams and Wilkins Co. Used by permission.)

hormone was administered by continuous diffusion from a Silastic implant, as developed by Dr. Pentti Siiteri and adapted by Dr. Jirayr Roubinian for use in the mouse (9). The implants used contain approximately 15 mg of hormone in a 2 cm piece of tubing. This produces sustained levels of β-estradiol that are at least 9 times as high as the mean normal level (assay kindly performed by Dr. William Crowley, Massachusetts General Hospital, Boston). The implants were placed subcutaneously when the mice were 4 weeks old, and for most of the studies the implants were left in for a total of 6 weeks.

Over the course of 6 weeks, sustained high levels of estrogen have pronounced effects on the mouse lymphoid and hematopoietic tissue. The thymus involutes and is barely detectable (10). At the endosteal surface of bone, new bone is induced with extensive replacement of the marrow (11). In response to the loss of hematopoietic tissue in the marrow, the spleen enlarges (12). There is also an increase in splenic macrophages (13). Because of these extensive effects of β-estradiol, one must be cautious in ascribing changes in the immune system to any one action of the hormone. Nevertheless, we suggest that estrogens may affect certain host responses, including natural killing, through their action on the bone marrow.

Figure 1 shows the effect of β-estradiol on natural killing by mice. Six weeks of estrogen substantially lowered natural killing in both males and females. Androgen (5α-methyltestosterone) had little effect (not shown). This effect of estradiol was not dependent on castration of mice; castration alone had no effect on natural killing in female B/W mice and lowered natural killing only slightly in males (Figure 1). Moreover, estradiol was as effective against natural killing in noncastrated mice as in castrated mice (Figure 1). This effect of estradiol on natural killing was seen in all strains of mice tested (DBA/2, Balb/c, CB7BL/6, and B10.D2) and in both sexes (data not shown).

The time course of this effect of estrogen was then studied. Two weeks after the implant, there was virtually no reduction in natural killing, but 4 weeks after the implant, natural killing was substantially reduced (Figure 2). The results shown are for male B/W mice, but similar results were obtained in female B/W mice and in C57BL mice of either sex. These mice were all implanted at 4 weeks of age, but similar results were obtained if the implant was placed at either 6 or 8 weeks of age and natural killing was measured at 10 weeks (i.e., 4 or 2 weeks of estrogen, respectively).

The implant was then removed after 6 weeks in order to observe the recovery of natural killing. Figure 3 shows that natural killing was still suppressed 2 weeks after the implant was removed. After 4 weeks there was partial recovery, and after 8 weeks, recovery was complete.

The delayed effect of estrogen on natural killing suggested that estrogens were not rapidly toxic to mature NK cells. This finding was confirmed by experiments in which estradiol was included in the assay for natural killing at concentrations up to 10 μg/ml. The inclusion of estradiol in the in vitro assay had no effect on natural killing. (The effects of prolonged incubation with estrogen could not be tested, because NK cells are rapidly inactivated in culture without estrogen.)

There was no evidence in estrogen-treated mice for a humoral or cellular suppressor of natural killing. Serum from estrogen-treated mice did not differ from normal mouse serum in its effect on natural killing (slight reduction by either), and spleen cells from estrogen-treated mice did not suppress killing by normal spleen cells (data not shown).

We considered the possibility that NK cells in the spleen were simply diluted by hematopoietic cells and/or macrophages that were increased in the spleens of estrogen-treated mice. Estrogen-treated mice did have enlarged spleens filled with red cells. However, the total number of white cells was, on the average, approximately 20% less than in spleens from sham-treated

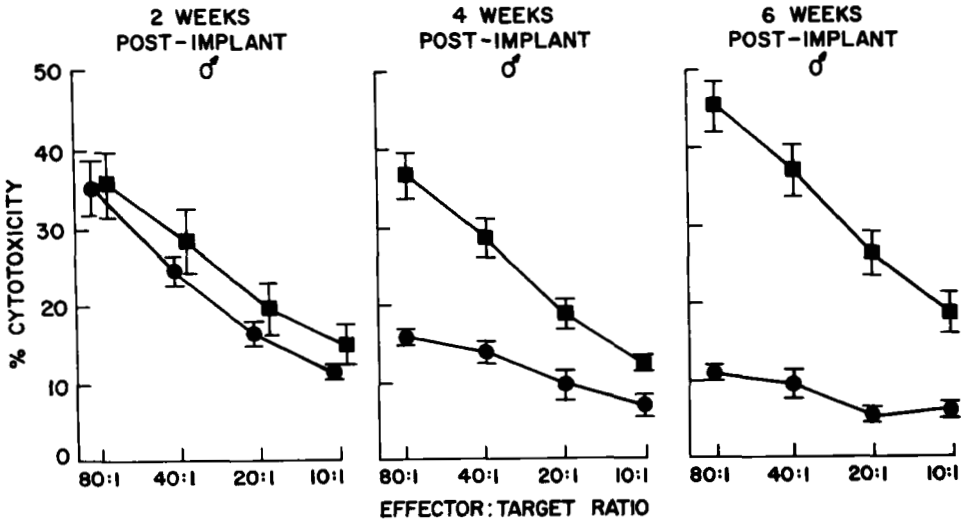

Figure 2. Time course of the effect of 17β-estradiol on natural killing by spleen cells from male NZB/NZW mice. Mice were treated with β-estradiol (circles) or with sham implant (squares). (From Seaman WE et al. J Immunol 121:2193–2198, 1978. Copyright 1978, The Williams and Wilkins Co. Used by permission.)

mice. Moreover, natural killing was shown to be decreased in the lymph nodes from estrogen-treated mice; lymph nodes are not a site of extramedullary hematopoiesis.

Since estrogen is not directly toxic to mature NK cells, and estrogen-treated mice do not have a cellular or humoral suppressor of natural killing, it is likely that estradiol is either toxic to the precursor of NK cells or detrimental to the production of NK cells from their precursors. If estradiol is toxic to NK precursors, the hormone is not completely lethal to the precursor cells, since natural killing recovers when the estradiol is dis-

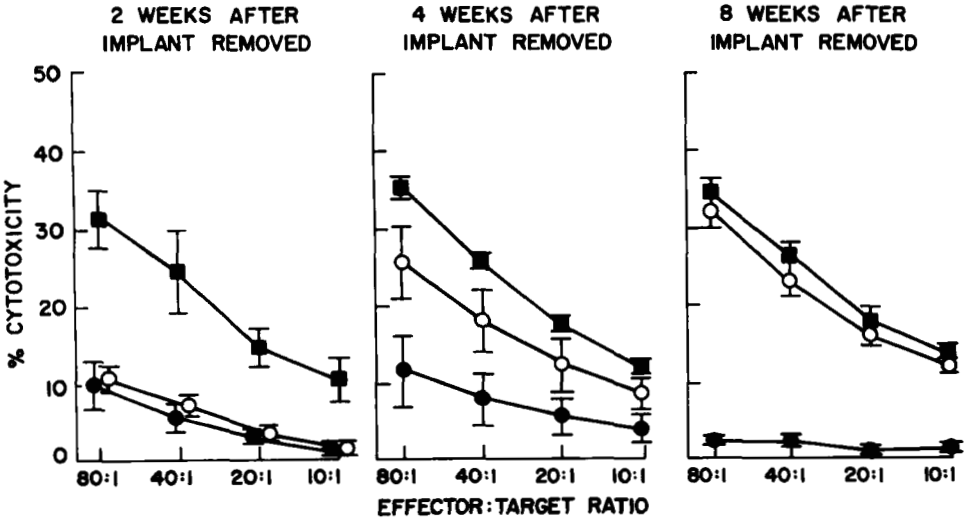

Figure 3. Recovery of natural killing by spleen cells from male NZB/NZW mice after 6 weeks of treatment with 17β-estradiol. Solid circles show natural killing by spleen cells from mice in whom β-estradiol was continued, open circles show killing by spleen cells from mice that received β-estradiol for 6 weeks and then had the implant removed. Solid squares show killing by mice that had a sham implant removed after 6 weeks. (From Seaman WE et al. J Immunol 121:2193–2198, 1978. Copyright 1978, The Williams and Wilkins Co. Used by permission.)

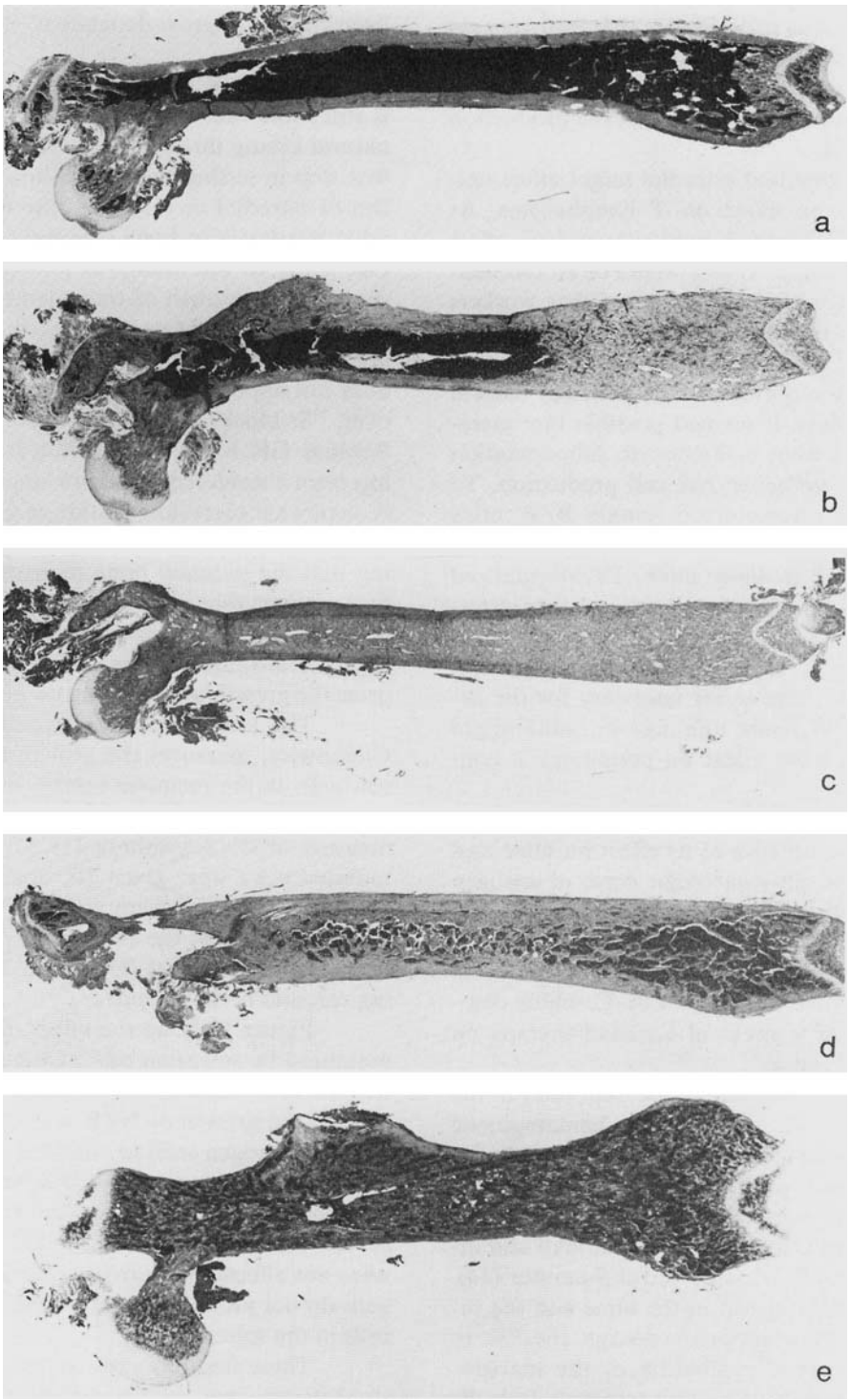

Figure 4. Histology of the femur in estrogen-treated and in congenitally osteopetrotic mice. Figures **a** through **d** show femurs (×5.3) from male NZB/NZW mice given an implant at 4 weeks: **a,** sham implant for 6 weeks (normal histology); **b,** estrogen implant for 2 weeks, showing partial replacement of the marrow cavity by new bone; **c,** estrogen implant for 8 weeks, showing virtually complete replacement of marrow by bone; **d,** estrogen implant for 6 weeks, then removed, femur 8 weeks after removal, showing partial recovery of marrow; **e,** femur from 5-week-old Grüneberg osteopetrotic (*mi/mi*) mouse, showing increased skeletal mass and reduced marrow (×13). (From Seaman WE et al. J Immunol 122:2541–2547, 1979. Copyright 1979, The Williams and Wilkins Co. Used by permission.)

continued. Although it is still possible that estrogens do directly affect the precursor of NK cells, the remainder of our studies have examined the possibility that estrogens alter the host in such a manner that the production of NK cells is blocked.

It was considered that estradiol might affect natural killing through an effect on T lymphocytes. As mentioned earlier, estrogens share the thymolytic effect of other steroid hormones. After 6 weeks of an estradiol implant, the thymus is difficult to find. Other workers have shown that the thymus is not essential to the production of NK cells, since nude (athymic) mice or thymectomized mice have normal (or heightened) natural killing (6). Nevertheless, it seemed possible that estrogen might selectively alter a thymocyte subpopulation in a way that would influence NK cell production. To examine this, we thymectomized female B/W mice within 24 hours of birth and studied the effects of estradiol on natural killing in these mice. Thymectomized mice had normal levels of natural killing. When estradiol was given at 4 weeks, natural killing at 10 weeks was markedly reduced, as in normal mice (data not shown). The thymus, then, is not necessary for the action of estradiol on NK cells, although estradiol might still work through a toxic effect on peripheral T lymphocytes.

A second consideration was that estrogen might reduce natural killing because of its effect on bone and bone marrow. In mice, pharmacologic doses of estrogen stimulate the generation of new bone at endosteal surfaces. The new bone intrudes on the marrow and eventually replaces it. The effect is particularly dramatic in the long bones, as shown in Figure 4, A–C, which demonstrates the effects of 6 weeks of estradiol therapy on the femurs from B/W mice.

The bone marrow appears to be important in the generation of mature NK cells. Unlike hematopoietic cells, which can migrate to the spleen when the marrow is injured, the precursors of NK cells apparently cannot. The evidence for this is based on the observation that natural killing in mice is sensitive to the in vivo administration of ^{89}strontium (^{89}Sr), a powerful β-emitter (14). A portion of the ^{89}Sr is trapped in the bone and the remainder is excreted. In appropriate dosage, the ^{89}Sr in the bone provides intense irradiation of the marrow, leading to a loss of marrow cells, with relatively little direct effect on the cells in the spleen, lymph nodes, or thymus (15). Treatment of mice with ^{89}Sr reduces natural killing, suggesting that the bone marrow is important in the generation of NK cells. NK cells have therefore been called "marrow-dependent" cells or "M" cells (14–16).

If the NK cells are marrow-dependent (the issue is still somewhat controversial), estrogens might reduce natural killing through their effect on the marrow. As a first step in testing this possibility, we examined the effect of estradiol on a second ^{89}Sr-sensitive function, genetic resistance to bone marrow transplantation (GR). GR refers to the ability of lethally irradiated mice to suppress the growth of transplanted foreign bone marrow cells (17,18). Mice that have been lethally irradiated are unable to generate cytotoxic T cells; therefore, GR does not appear to be dependent on these cells. Moreover, ^{89}Sr blocks GR without blocking T cell immunity. Because GR is sensitive to ^{89}Sr, it, like natural killing, has been considered a marrow-dependent function (16). A somewhat surprising feature of GR is the ability of F_1 mice to reject marrow cells from either parent, suggesting that the parental bone marrow cells carry cell surface markers that differ from those in their heterozygous offspring (18). This feature of GR was used to test the effect of estradiol on the ability of B/W mice to suppress the growth of bone marrow cells from NZB mice.

The assay for GR, developed by Dr. Gustavo Cudkowicz, measures the growth of transplanted marrow cells in the recipient spleen, using the uptake of 5-iodo-2-deoxuridine (IUdR), a thymidine analog, as a measure of DNA synthesis (19,20). In brief, lethally irradiated mice were given 10^7 bone marrow cells. Four days later the mice were given IUdR labeled with ^{125}I. The following day the entire spleen was removed to determine the uptake of IUdR into DNA in newly forming colonies in the spleen.

Figure 5 shows the effect of estradiol on GR as measured by retention of ^{125}IUdR in the spleen. Sham-treated B/W mice (mice given an empty implant) suppressed the growth of NZB marrow cells in the spleen, whereas estrogen-treated mice did not; the replication of NZB cells in estrogen-treated B/W mice was similar to the growth of NZB cells in syngeneic NZB mice. Syngeneic transfers (NZB into NZB or B/W into B/W) were not affected by estrogen, demonstrating that estrogens do not promote a nonspecific increase in growth of cells in the spleen.

These findings were consistent with the hypothesis that estrogens reduce natural killing by altering the bone marrow. Since the gross effect of estrogen on the bone marrow involves the overgrowth of marrow by bone, we examined whether natural killing might be reduced in another situation where marrow is replaced by

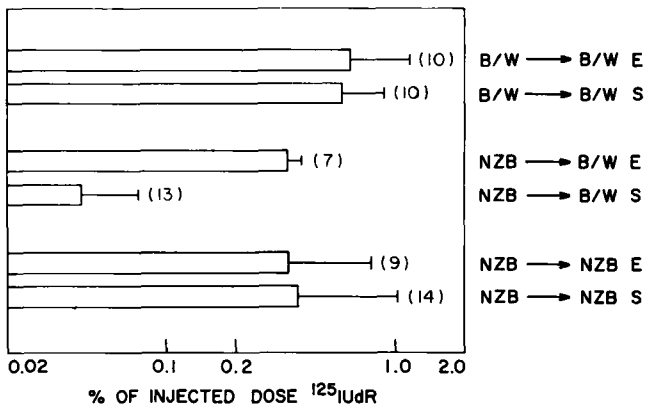

Figure 5. Genetic resistance to parental (NZB) marrow transplantation by NZB/NZW mice that received either estrogen (E) or sham (S) implant for 6 weeks. Bars show percent ^{125}I retained in the spleen 16–18 hours after injection of $^{125}IUdR$ into mice that had been irradiated and given 10^7 bone marrow cells 4 days previously. The number of mice assayed for each transfer is shown in parentheses. Controls include transplantation of NZB/NZW cells into syngeneic recipients (top two bars) and transplantation of NZB cells into syngeneic recipients (bottom two bars). (From Seaman WE et al. J Immunol 122:2541–2547, 1979. Copyright 1979, The Williams and Wilkins Co. Used by permission.)

bone—congenital osteopetrosis. There are several mouse models for congenital osteopetrosis (21). We used mice homozygous for microphthalmia (*mi*), a recessive gene on the 6th chromosome that leads to osteopetrosis in utero (Figure 4E). When mice were tested at 4 weeks, all of 7 CBA (*mi/mi*) mice had deficient natural killing compared to their normal littermates (Figure 6). This deficiency was also seen in 7 of 9 Grüneberg (*mi/mi*) mice, although 1 *mi/mi* mouse had normal levels of natural killing and 1 had high levels (Figure 6).

Again, these findings are consistent with the hypothesis that alterations in the bone marrow affect NK cells. We therefore conclude that estradiol may affect NK cells by altering the bone marrow. However, this effect does not appear to simply result from a reduction in the amount of bone marrow, since analysis of the bone marrow volume in estrogen-treated mice revealed that natural killing was lost more rapidly than the total marrow volume, and that natural killing recovered more rapidly than the marrow volume when estrogens were discontinued (data not shown).

In summary, our working hypothesis is that estrogens lower natural killing by altering the bone marrow. This hypothesis is unproved but fits the present findings.

Dr. J. R. Roubinian and others have shown that

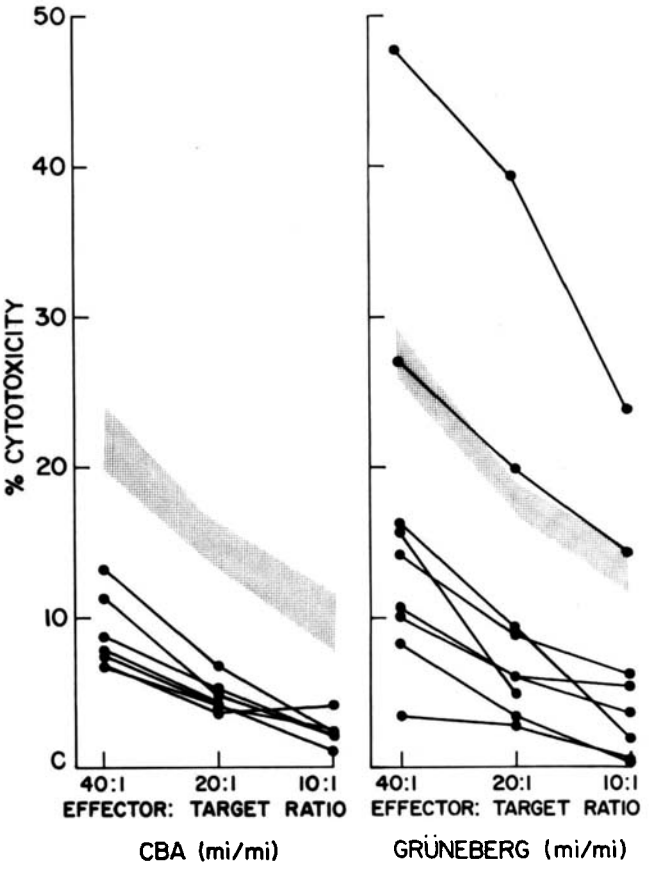

Figure 6. Natural killing by spleen cells from 4-week CBA (*mi/mi*) mice (left) and from 5-week Grüneberg (*mi/mi*) mice (right). Solid lines show results for individual (*mi/mi*) mice. The shaded area is the mean ±SEM for their normal littermates. (From Seaman WE et al. J Immunol 122:2541–2547, 1979. Copyright 1979, The Williams and Wilkins Co. Used by permission.)

estrogens exacerbate autoimmunity. Is it possible that this effect is mediated by the effect of estrogen on NK cells? We do not think that this is the case. When natural killing is lowered in B/W mice by the in vivo administration of ^{89}Sr, we find a reduction in autoimmunity rather than an increase (22). ^{89}Sr is probably more selective in its effect on NK cells than is estrogen. It therefore seems likely that estrogen increases autoimmunity through a mechanism that does not directly involve NK cells. In fact, a reduction in NK cells may actually improve autoimmune disease.

ACKNOWLEDGMENTS

These studies were carried out in the laboratory of Dr. Norman Talal, with the collaboration of Drs. John Loeb, John Greenspan, Jirayr Roubinian, and Ms Marcia Blackman.

REFERENCES

1. Seaman WE, Blackman MA, Gindhart TD, Roubinian JR, Loeb JM, Talal N: β-estradiol reduces natural killer cells in mice. J Immunol 121:2193–2198, 1978
2. Seaman WE, Gindhart TD, Greenspan JS, Blackman MA, Talal N: Natural killer cells, bone, and the bone marrow: studies in estrogen-treated mice and in congenitally osteopetrotic mice. J Immunol 122:2541–2547, 1979
3. Welsh RM Jr: Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. J Exp Med 148:163–181, 1978
4. Lopez C: Genetic resistance to HSV-1 in the mouse is mediated by the "M" cell of allogeneic resistance (abstract). Fed Proc 37:1560, 1978
5. Herberman RB, Nunn ME, Holden HT, Laurin DH: Natural cytotoxic activity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer 16:230–239, 1975
6. Kiessling R, Klein E, Pross N, Wigzell H: Natural killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells: characteristics of the killer cell. Eur J Immunol 5:117–121, 1975
7. Hansson M, Kiessling R, Andersson B, Kärre K, Roder J: NK cell-sensitive T-cell subpopulation in thymus: inverse correlation in host NK activity. Nature 178:174–176, 1979
8. Herberman RB, Nunn ME, Holden HT: Low density of Thy-1 antigen on mouse effector cells mediating natural cytotoxicity against tumor cells. J Immunol 121:304–309, 1978
9. Roubinian JR, Talal N, Greenspan JS, Goodman JR, Siiteri PK: Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F_1 mice. J Exp Med 147:1568–1583, 1978
10. Dougherty TF: Effect of hormones on lymphatic tissue. Physiol Rev 32:379–401, 1952
11. Silberg M, Silberg R: Steroid hormones and bone, The Biochemistry and Physiology of Bone. Vol. 3. Second edition. Edited by G Bourne. New York, Academic Press, 1971, pp 401–486
12. Fried W, Tichler T, Dennenberg I, Barone J, Wang F: Effects of estrogens on hematopoietic stem cells and on hematopoiesis of mice. J Lab Clin Med 83:807–815, 1974
13. Vernon-Roberts B: The effects of steroid hormones on macrophage activity. Int Rev Cytol 25:131–158, 1969
14. Haller O, Wigzell H: Suppression of natural killer cell activity with radioactive strontium: effector cells are marrow dependent. J Immunol 118:1503–1506, 1977
15. Bennett M, Baker EE, Eastcott JW, Kumar V, Yonkosky D: Selective elimination of bone marrow precursors with the bone seeking isotope ^{89}Sr: implications for hemopoiesis, lymphopoiesis, viral leukemogenesis and infection. J Reticuloendothel Soc 20:71–87, 1976
16. Bennett M: Prevention of marrow allograft rejection with radioactive strontium: evidence for marrow-dependent effector cells. J Immunol 110:510–516, 1973
17. Cudkowicz G, Bennett M: Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice. J Exp Med 134:83–102, 1971
18. Cudkowicz G, Bennett M: Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F_1 hybrid mice. J Exp Med 134:1513–1528, 1971
19. Cudkowicz G, Upton AC, Smith LM, Gosslee DG, Hughes WL: An approach to the characterization of stem cells in mouse bone marrow. Ann NY Acad Sci 114:571–582, 1964
20. Bennett M, Cudkowicz G, Foster RS Jr, Metcalf D: Hemopoietic progenitor cells of *W* anemic mice studied in vivo and in vitro. J Cell Physiol 71:211–226, 1974
21. Marks SC Jr, Walker DG: Mammalian osteopetrosis—a model for studying cellular and humoral factors in bone resorption, The Physiology and Biochemistry of Bone. Vol. 4. Second edition. Edited by G Bourne. New York, Academic Press, 1971, pp 227–301
22. Seaman WE, Blackman MA, Greenspan JS, Talal N: ^{89}Strontium reduces autoimmunity in NZB/NZW mice (abstract). Arthritis Rheum 22:657, 1979