Role of Suppressor Cells in the Decline of Natural Killer Cell Activity in Estrogen-treated Mice

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ABSTRACT

Previous studies have shown that prolonged estrogen treatment of mice markedly reduces their natural killer (NK) cell activity. Our experiments demonstrate that splenocytes from (C57BL/6 × C3H/He) F₁ mice treated with 17β-estradiol are suppressive for the NK activity of splenocytes from untreated mice when the two cell populations are mixed during cytotoxicity assays. The suppressor activity is resistant to treatment with anti-Thy-1.2 or anti-la reagent plus complement, can be generated in neonatally thymectomized mice, and is present in plastic-adherent as well as nonadherent cell populations. Treatment with a NK-reactive antiserum, either anti-asiado-GM-1 or anti-NK-1,2, has no effect on the suppressor activity. Administration of the interferon inducer polyinosinic-polycytidylic acid to mice treated with estrogen results in moderate restoration of NK activity but has no effect on the suppressor activity. These data suggest that generation of a Thy-1-negative/la-negative suppressor cell population is, at least in part, responsible for the reduced levels of NK activity in estrogen-treated mice.

INTRODUCTION

Sustained exposure of mice to estrogen has profound effects on the histology and function of hematopoietic tissues; the thymus atrophies (5), some of the marrow is replaced by bone (30), and the spleen enlarges, due mainly to erythropoiesis (7). Extensive studies by Seaman et al. (28, 30, 31), Seaman and Gindhart (29), and Seaman and Talal (32) revealed that the mice thus treated have substantially reduced levels of NK³ activity 4 weeks after initiation of the treatment. The effect of estrogen was reversible; NK activity was completely restored 8 weeks after termination of the treatment (29). Since the decline in NK activity preceded the loss of marrow (30), it has been suggested that the reduced levels of NK activity result from altered cell population dynamics in marrow-dependent NK progenitors (30).

NK activity is modulated by a variety of experimental conditions and reagents (2-4, 8-16, 20, 23, 24, 26-32, 35) as well as by intrinsic factors (2, 17, 19, 25). Active suppression of NK-mediated cytolyis by suppressor cells has been implicated in some cases as the principal mechanism for negative regulation (2, 4, 15, 16, 20, 26, 27). The data presented below suggest that the presence of a regulatory cell population is, at least in part, responsible for the reduced levels of NK activity in mice treated with estrogen.

MATERIALS AND METHODS

Mice. Female (C57BL/6 × C3H/He) F₁ mice (hereafter called B6C3F₁) were obtained from the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and were used at the age of 5 to 7 weeks. Pregnant female C57BL/6 mice crossed with C3H/He males were also obtained from the same source, and the newborn F₁ mice were used for neonatal thymectomy.

Tumor Cells. YAC-1 lymphoma cells of A/Sn origin were maintained in spinner culture in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 5% heat-inactivated bovine serum. YAC-1 cells used for NK assays were in the log phase of the growth curve.

Irradiation. Mice were exposed to 700 rads of total-body γ-irradiation at the rate of 112 rads/min using a 125Cs source (Gammasell-20 Small Animal Irradiator; Atomic Energy of Canada, Ltd., Ottawa, Canada).

Thymectomy. Neonatal thymectomy was performed within 24 hr postpartum as described previously (34).

Plastic Adherence. Enrichment and recovery of plastic-adherent splenocytes was as described previously (6). This procedure was slightly modified in that an incubation period of 2 hr was used.

Estrogen Treatment. Sealed silastic tubes and tubes containing 17β-estradiol were implanted into 6-week-old mice s.c. along the lower spine as described previously (28). The estrogen tubes, as well as the empty tubes for sham implants, were kindly provided by Dr. William E. Seaman, Ft. Miley Veterans Hospital, San Francisco, Calif.

Administration of poly(I)-poly(C). Mice were given i.p. injections of 100 µg of poly(I)-poly(C) (Sigma Chemical Co., St. Louis, Mo.) in 0.1 ml of balanced salt solution 18 hr before the NK assay.

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Treatment with Antibodies and Complement. Tissue culture supernatant of rat/mouse hybridoma clone 30-H12 (obtained from Dr. N. L. Warner, Cell Distribution Center, University of New Mexico School of Medicine) was the source of anti-Thy-1.2 antibodies. Hybridoma clone 10-2.16 (obtained from the Cell Distribution Center, The Salk Institute, San Diego, Calif.) provided antibodies against mouse la antigens. One ml of anti-Thy-1.2 reagent was used for 1 × 10⁶ cells, and 1 ml of anti-la was used for 5 × 10⁶ cells; both reagents were added to the cells at the final dilution of 1:2. RC (Grand Island Biological Co.) was used at a final dilution of 1:15. Details of the procedure have been described previously (15). Under these conditions, the anti-la reagent plus RC inactivated splenic adherent cells serving as stimulator and accessory cells for in vitro induction of cell-mediated lympholysis (6). To ascertain the efficacy of anti-Thy-1.2 reagent and RC, allogeneic cytotoxic T-lymphocytes were treated simultaneously with the same reagents and assayed on appropriate lymphoma target cells as described previously (22). Anti-asiado-GM-1 was generously provided by Dr. T. Tada, University of Tokyo, Japan, and was used at a final dilution of 1:40, 1 ml/2 × 10⁶ cells, plus RC (Grand Island Biological Co.). Anti-NK-1.2 serum (CE anti-CBA) and prescreened RC were a generous gift of Dr. Robert G. Burton, University of Newcastle, Newcastle, Australia, and was used as described (1).

Assay for NK and Suppression of NK. Splenic NK activity and in vitro suppression of NK activity were determined in a 4-hr ⁵¹Cr release assay using YAC-1 lymphoma cells as targets as described previously (13, 20). Suppression assays were performed at the effector:target cell ratio of 100:1.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NK, natural killer; poly(I)-poly(C), polyinosinic-polycytidylic acid; RC, rabbit complement.
ratio of 100 (2 × 10⁶ effector cells and 2 × 10⁶ target cells) in the presence of graded numbers of suppressor cells. Suppression assays and direct NK assays were performed simultaneously, using the same cell preparations. Each suppression assay included positive and negative controls; splenocytes from irradiated mice (700 rads of γ-rays 15 to 21 days earlier) were the source of suppressors for the positive control (14), and thymocytes were used as fillers (negative control) to monitor the effect of cell crowding, since thymocytes are inactive against YAC-1 target cells. Splenic NK activity was determined for individual animals, except in experiments in which cells from 3 to 8 spleens were pooled for treatment with antisera and RC.

RESULTS

Seven weeks after implantation of silastic tubes containing estrogen or control tubes without estrogen, individual mice were tested for splenic NK activity as well as for suppressor activity against NK-mediated cytolysis. Some of the mice with estrogen implants were given an i.p. injection of poly(I)-poly(C) 18 hr prior to the NK and suppressor assays. The results from several experiments are pooled and presented in Chart 1. NK activity of mice treated with estrogen was markedly reduced at all effector:target ratios (Chart 1, left). Injection of poly(I)-poly(C) resulted in moderate restoration of the NK activity in these mice. In contrast, NK levels of mice with sham implants were consistently high and could be elevated to still higher levels by poly(I)-poly(C) treatment.

Suppressive function of spleen cells from estrogen-treated mice was tested in vitro against NK activity of spleen cells from normal mice that had been stimulated with poly(I)-poly(C). Splenocytes from mice with estrogen implants, with or without poly(I)-poly(C) treatment, invariably suppressed the NK-mediated cytolysis. However, the suppressive activity of splenocytes from mice treated with estrogen was considerably weaker than that

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Suppressor cells</th>
<th>% specific lysis</th>
</tr>
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<tbody>
<tr>
<td>Untreated splenocytes</td>
<td>Thymocytes</td>
<td>62.7 ± 5.1</td>
</tr>
<tr>
<td>Estrogen-treated splenocytes</td>
<td>Estrogen-treated thymocytes</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>Estrogen-treated splenocytes + medium</td>
<td>Estrogen-treated thymocytes + medium</td>
<td>3.6 ± 1.0</td>
</tr>
<tr>
<td>Estrogen-treated splenocytes + RC</td>
<td>Estrogen-treated thymocytes + RC</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>Estrogen-treated splenocytes + anti-Thy-1.2</td>
<td>Estrogen-treated thymocytes + anti-Thy-1.2</td>
<td>4.3 ± 1.5</td>
</tr>
<tr>
<td>ESTrogen-treated splenocytes + anti-la</td>
<td>2.2 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Untreated splenocytes</td>
<td>Estrogen-treated thymocytes</td>
<td>56.3 ± 6.4</td>
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<tr>
<td>Untreated splenocytes</td>
<td>Estrogen-treated thymocytes</td>
<td>17.8 ± 3.5</td>
</tr>
<tr>
<td>Untreated splenocytes</td>
<td>Estrogen-treated thymocytes + medium</td>
<td>23.5 ± 5.5</td>
</tr>
<tr>
<td>Untreated splenocytes</td>
<td>Estrogen-treated thymocytes + RC</td>
<td>20.5 ± 4.2</td>
</tr>
<tr>
<td>Untreated splenocytes</td>
<td>Estrogen-treated thymocytes + anti-Thy-1.2 + RC</td>
<td>22.8 ± 4.8</td>
</tr>
<tr>
<td>Untreated splenocytes</td>
<td>Estrogen-treated thymocytes + anti-la + RC</td>
<td>7.0 ± 3.5</td>
</tr>
</tbody>
</table>

* Assayed at the effector:target ratio of 100:1.
* Four million cells were added to a mixture of 2 × 10⁶ untreated spleen cells and 2 × 10⁶ target cells.
* Results of 3 to 5 replicate experiments were pooled.
* Mean ± S.E.
* p < 0.05 when compared to splenocytes from mice with estrogen implants and exposed to anti-Thy-1.2 plus RC.

of the positive control for suppression; i.e., spleen cells from mice exposed to 700 rads of γ-irradiation 15 to 21 days earlier (Chart 1, right).

Neonatal thymectomy had little effect on direct splenic NK activity of mice with estrogen implants (Chart 1, left), but splenic suppressor activity was stronger in these thymectomized mice than in nonthymectomized mice with estrogen implants (Chart 1, right). Treatment of splenocytes from mice carrying estrogen implants with anti-Thy-1.2 or anti-la reagent plus RC neither restored the NK activity nor removed the suppressive activity (Table 1); depletion of la-positive cells from the spleen resulted in enhancement of suppressive activity (Table 1). Anti-NK-1.2 or anti-asialo-GM-1 plus RC had no effect on the suppressive activity of splenocytes from estrogen-treated mice (Table 2).

Separation of splenocytes from mice treated with estrogen into plastic-adherent and nonadherent fractions resulted in suppressor cells for NK present in both cell populations. Adherent and nonadherent splenocytes from mice treated with estrogen were equally effective in suppressing NK activity of normal splenocytes, whereas adherent splenocytes from untreated mice, although showing reduced NK activity, had no suppressive effect (Table 3). The direct NK activity of the separated cell fractions also remained low (Table 3).

DISCUSSION

Our data demonstrated the presence of suppressor cell activity against NK-mediated cytolysis in the spleens of mice with estrogen implants. The suppressor activity was not borne by a Thy-1-positive cell, nor was the induction of such activity dependent on the thymus. Exposure of suppressive splenocytes to anti-la reagent and RC enhanced the suppressor activity,
presumably due to enrichment through depletion of nonactive cells. The suppressor activity for NK was present in the plastic-adherent and plastic nonadherent cell populations. Therefore, the possible existence of 2 distinct classes of suppressor cells is indicated. Treatment of the suppressor-containing spleen cells with anti-NK sera did not remove the suppressor activity, providing evidence against the possibility that the suppressor cells are noncytolytic NK cells competing with the YAC-1 targets.

Administration of poly(I)-poly(C) to mice with estrogen implants results in substantial levels of circulating interferon (31), a potent, positive regulator of NK activity (10, 11, 33, 35). Treatment with poly(I)-poly(C) significantly increased the NK activity of splenocytes from mice with estrogen implants when tested in direct NK assay, although the levels of NK remained well below those found in mice with sham implants and given the same dose of poly(I)-poly(C) (Chart 1, left). These data indicate that NK cells and/or their progenitors were not entirely depleted from the spleen by the estrogen treatment. Since poly(I)-poly(C) administration did not weaken the suppressor cell activity (Chart 1, right), poly(I)-poly(C)-dependent augmentation of NK activity in estrogen-treated mice appears to represent genuine activation or recruitment, rather than a release of preexisting activity from suppression.

A recent study showed that NK activity of normal spleen cells can be adaptively transferred to mice which were treated with estrogen and that the transfer resulted in reduced hematogenous tumor metastasis in the recipients (9). The authors also showed, as did the present study, that NK activity of mice treated with estrogen can be significantly increased by poly(I)-poly(C) stimulation. Since residual NK cells and/or their progenitors in the estrogen-treated mice are capable of responding to poly(I)-poly(C) stimulation and show moderate levels of activity in spite of coexisting suppressive influence, it is not surprising that adaptively transferred normal spleen cells manifest moderate levels of NK activity in the recipient.

The discrepancy between the reports by Seaman et al. (28, 30, 31), Seaman and Gindhart (29), and Seaman and Talal (32) and our findings with respect to the presence of active NK suppression in mice with estrogen implants is somewhat puzzling. We chose to use B6C3F1 mice because of their naturally high levels of splenic NK activity, whereas Seaman et al. (28, 30, 31), Seaman and Gindhart (29), and Seaman and Talal (32) used BALB/c and NZB/NZW mice. Genetic background does play a role in the expression of NK activity (17, 19, 25) and may also be important in its regulation. Although Seaman et al. (32) used the suppression assay of Hochman and Cudkowicz (13), there may be a difference in the details of methodology. Recently, we have reported on the importance of absolute numbers of suppressors added to the effectors, rather than suppressor:effector cell ratios, in demonstrating the suppressor activity induced by Corynebacterium parvum (20). Furthermore, Seaman et al. (28, 30, 31), Seaman and Gindhart (29), and Seaman and Talal (32) used NH4Cl to lyse RBC in the splenocyte suspensions, whereas we did not treat the splenocytes in any way. Although RBC have little effect on suppression, as demonstrated by the suppressive activity of plastic-adherent erythrocyte-free splenocytes from estrogen-treated mice (Table 3), suppressor cells may be sensitive to treatment with NH4Cl. We, as well as Hanka and Schneider (9), observed a significant increase of NK activity following poly(I)-poly(C) treatment of mice with estrogen implants, but Seaman et al. (31) did not see such a response, although they reported that the foot-pad injection of herpes simplex virus did increase NK activity in estrogen-treated mice (32). In addition to the difference in strains of mice used, the dose of poly(I)-poly(C) we used was 100 µg/mouse i.p., while Seaman et al. (31) used 50 µg i.v. The route of administration of various reagents has been reported to affect the response in animals (23, 24).

The bone marrow origin of NK cells is well established (8, 18, 21). The possibility remains open, however, that some of the progenitor cells for NK reside, differentiate, and mature in other organs, especially when hemopoiesis is not maintained by the bone marrow. Although estrogen treatment leads to osteoporosis in mice, NK activity is reduced before detectable changes occur in the marrow volume (30). Persistently high levels of estrogen may cause imbalance in the existing marrow cell populations, leading to excessive function of certain cell popu-
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REFERENCES

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