Interaction between Thymosin, Testosterone and Estradiol on Natural Killer Cell Activity in Mice

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DESCHAUX, P., PAUCOD, J.-C. and ARDAIL, D. *Interaction between Thymosin, Testosterone and Estradiol on Natural Killer Cell Activity in Mice.* Tohoku J. exp. Med., 1982, 136 (4), 367-372 — Thymosin fraction 5, an immunopotentiating thymic preparation, significantly increases the cytotoxic capacity of NK cells isolated from the spleen. This stimulation is inhibited by testosterone and estradiol. — — — thymosin; natural killer cells; spleen; testosterone; estradiol

Natural killer (NK) cells are lymphocytes that rapidly kill certain tumors and transformed cells in vitro despite a lack of known sensitization to the target. Natural cytotoxicity can be augmented by a number of environmental agents, including tumor targets themselves, a variety of viruses, adjuvants and interferon (Clark et al. 1979). Today accumulated data support the hypothesis that cancer patients with relatively low levels of cellular immunity who undergo immunosuppressive therapy would benefit most from administration of thymosin, a family of thymic hormones. Thymosin effects in cancer patients might be due to increase in mature T cells which would then enhance endogenous antitumor activity. In addition, thymosin may function in immunosuppressed cancer patients to increase T cell reactivity and thus help in reducing the high incidence of infection that often accompanies treatment radiotherapy and chemotherapy (Low and Goldstein 1979). In this perspective, we have been trying to demonstrate an action of thymosin on NK cells which might be pre-T lymphocytes according to some authors (Herberman and Holden 1978; Bardos et al. 1979). NK activity does not differ between male and female mice, but the effect of sex hormones on natural killing has not otherwise been investigated. Recently, Seaman et al. demonstrated that pharmacologic amounts of estrogens, given chronically, will substantially lower NK activity in the mouse (Seaman et al. 1978). In previous works, we demonstrated that testosterone and estrogens inhibited the immunostimulation of antibody production initiated by thymosin (Deschaux et al. 1979a, 1980). Therefore, we tested action of sexual steroids (testosterone and estradiol) directly on natural killer cells and in mixture with thymosin.

Received for publication, June 4, 1981.

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MATERIALS AND METHODS

Animals

Male and female Swiss mice were purchased from IFFA Credo Laboratories. All mice used for experiments were between 2 and 3 months of age.

Medium

Cell preparations were carried out in RPMI 1640 with 25 mM HEPES buffer plus 10% fetal calf serum (RPMI 10). For the NK assay, fetal calf serum was increased to 20% and the medium was supplemented with 2 mM glutamine, 50 μ g/ml penicillin G, 100 μ g/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol (RPMI 20).

Effector cells

Effector cells were obtained from Swiss mice killed by cervical dislocation. Spleen cells were prepared by mincing the spleen and teasing cells through 40 mesh steel screen. Red cells were lysed by incubation in 0.155 M ammonium chloride for 5 min in ice using bicarbonate buffer, pH 7.4, supplemented with 10% fetal calf serum. Cell suspensions were prepared in RPMI 10. For removal of T cells, akr-anti Thy 1.2.C₃H antibody was purchased from Flobio Laboratories. The treatment of lymphoid cells was performed according to the technique described by Glaser (1979). The antiserum, plus complement, lysed more than 30% of Swiss spleen cells, as determined by trypan blue dye exclusion. The T cell response of spleen cells to PHA was abolished after treatment, whereas the B cell response to LPS was left intact. For removal of B cells and macrophages, the technique with nylon wool columns described by Julius et al. (1973) was used. The lymphoid cells treated or not with anti Thy 1 antibody were cultured in a 2 ml volume of RPMI 20 in 24-well culture plates at 37°C in a humidified incubator (5% CO₂; 95% air) during 5 days. Testosterone (Calbiochem), estradiol (Calbiochem), thymosin fraction 5 (Hoffmann La Roche) or testosterone+thymosin, or estradiol+thymosin were added to obtain a final concentration of 10 μ g for each hormone. An oil solvent was used as a control for the steroids and physiologic saline for thymosin fraction 5. Triplicate cultures were performed for each hormone. All experiments were performed in twenty and the results averaged.

Target cells

The target for natural killer cells was a murine malignant melanoma (Villejuif) that we carry in vivo by subcutaneous inoculations into Swiss mice. Subcutaneous inoculations of up to 1.5×10^7 melanoma cells produced progressive tumor growth and eventually killed the animals. Mice are inoculated in the right flank. The tumor cells were collected and dispersed in the same way as the lymphocytes. In the chromium-51 assay, melanoma cells were labelled. These cells were adjusted to a concentration of 10^6 cells/0.8 ml RPMI. One tenth ml of Na⁵¹Cr₂O₄ solution at 2.0 mCi/ml (CEA Saclay France) was added to the melanoma cells and the mixture was incubated at 37°C for 45 min. The cells were washed three times in PBS, counted and adjusted to 10^5 cells/ml in RPMI 10 for use in the ⁵¹Cr release NK assay.

⁵¹Cr release assay

At the end of the 5 day lymphocyte culture period, cells from triplicate cultures were pooled, centrifuged and resuspended in RPMI 20 at a concentration of 10^6 living cells/ml; 0.1 ml aliquots of this cell suspension were pipetted into each of three wells of a microtest plate. At each culture we added 0.1 ml of target cells labelled with ⁵¹Cr. The cell mixtures were incubated for 4 hr at 37°C in a CO₂ incubator. After this incubation, the plates were centrifuged at 350 g for 5 min. One tenth ml of the supernatant was removed for counting. Results were calculated from the mean of triplicate samples and expressed as:

$$\text{Per cent lysis} = \frac{\text{cpm } ^{51}\text{Cr released in presence of spleen cells} - \text{cpm } ^{51}\text{Cr spontaneous release}}{\text{cpm } ^{51}\text{Cr released in presence of 1\% SDS} - \text{cpm } ^{51}\text{Cr spontaneous release}} \times 100$$

RESULTS

Effect of thymosin fraction 5 on the natural killer cells

The data presented in Fig. 1 show that thymosin (10 μ g) increases cytolytic activity of natural killer cells. Without treatment, the NK cells have a cytolytic effect equal to 8%, whereas after treatment the cytotoxicity is about 58% ($p < 0.001$). The treatment with anti-Thy 1 antibody and filtration on nylon wool has no effect, demonstrating that effector cells are NK cells.

Fig. 1. Effect of thymosin (10 μ g) on NK cell activity. The effect of anti-Thy 1 antiserum is tested with NaCl or with thymosin. † $p < 0.001$ vs. control. Each value is the mean $\pm$ S. E.

Effect of testosterone and estradiol

The effect of adding testosterone and estradiol directly to the cultures is reported in Fig. 2. Addition of oil (solvent for the steroids) has no effect. The increase of the cytotoxicity obtained with testosterone is significant; the decrease observed after treatment with estradiol is also significant ($p < 0.01$). In Fig. 3, we report the data concerning the effect obtained with thymosin+steroids. The

Fig. 2. Effect of testosterone (10 μ g) or estradiol on NK cell activity. * $p < 0.01$ vs. control. Each value is the mean $\pm$ S. E.

incubation with steroids significantly inhibits the stimulation of NK cells, obtained after treatment with thymosin alone. Estradiol appears to be more effective than testosterone.

Fig. 3. Effect of testosterone and estradiol on NK cells incubated with thymosin.

* $p < 0.01$ vs. thymosin. The mean $\pm$ S.E. is shown.

DISCUSSION

Within the lymphoid system of normal healthy mammals there exists a naturally occurring population of cytotoxic cells (Clark et al. 1979). These unconventional natural killer cells are distinct from macrophages and B lymphocytes. A number of reports suggest that NK cells could be pre-T cells (Bardos et al. 1979). In order to test this hypothesis, we decided to study the effect of thymosin fraction 5 on this cellular population. Thymosin fraction 5 was prepared from calf thymus as described previously by Hooper et al. (1975). Thymosin is a potent immunopotentiating preparation and can act in lieu to the thymus gland to reconstitute immune functions in certain thymus deprived and/or immunodepressed individuals. Thymosin has been found to induce T cell differentiation from pre-T lymphocytes (Low and Goldstein 1979) and enhance immunological function (Deschaux et al. 1980). Its secretion appears to be controlled by the endocrine system (Deschaux et al. 1979b). An important contribution of thymic hormones resides in their potential application to the clinical management of thymus dependent diseases. Data from several laboratories suggest that thymosin has a major role in restoring immune responsiveness and augmenting specific lymphocyte activities in patients with primary and secondary T-cell deficiencies resulting from a variety of disorders, including cancer (Kenady et al. 1977; Lipson et al. 1979). The first part of our study demonstrates that thymosin fraction 5 significantly increases the cytolytic effect of NK cells against tumor target cells, i.e. melanoma cells. The effect of thymosin was seen after treatment of lymphoid cells with anti-thy 1 antibody and filtration through nylon wool columns to eliminate T and B cells. Recently Patt and colleagues (Patt et al. 1979) have demonstrated that immunoincompetent melanoma patients treated with a low dose

of thymosin (4 mg/m²) in vivo showed a beneficial effect of hormonal therapy and suggested a role for thymosin on the T lymphocyte. Our results indicate that one effect of thymosin could be to potentiate the cytolytic effect of NK cells on melanoma cells. This result is in agreement with the recent data of Bardos et al. who demonstrated the same type of effect on NK cells using another thymic hormone, FTS (Bardos et al. 1979).

Many clinically important immune reactions are the sum of a number of different immune factors working either synergistically or antagonistically. Attention has been given to those situations in which other specific and non-specific factors can interact with immune factors. In previous works, we reported that thymosin increased the plaque forming cell (PFC) response to sheep red blood cells in spleen cell cultures. This stimulation, obtained after treatment with thymosin, is decreased when thymosin is added in a mixture with testosterone or estradiol (Deschaux et al. 1979a, 1980).

The second part of our study deals with the effect of testosterone or estradiol on modulating the cytolytic effect of NK cells. Fig. 2 shows that testosterone has direct effect on NK cells; a significant decrease is obtained with estradiol. The data reported in Fig. 3 show that the two sexual steroids inhibit the stimulation obtained with thymosin. The hormone concentrations used in these experiments are the same as reported in previous studies (Deschaux et al. 1979a, 1980). The decrease of cytotoxicity of NK cells obtained with estrogen confirms that data of Seaman and colleagues (1978), who demonstrated that pharmacological amounts of estrogen given chronically will substantially lower NK activity in the mouse. We have also confirmed previous reports that NK activity does not differ between male and female mice (percentage of cytotoxicity=8%).

The interaction and antagonism between thymosin and these gonadal steroids would suggest the importance of determining the plasma levels of testosterone and estradiol in patients with immunodeficiencies who do not respond to thymosin.

Acknowledgments

This work was supported by a NATO grant. We thank Dr. J.L. Touraine for his constructive advice, and Dr. A.L. Goldstein, for critical reading of the manuscript.

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