Immunology Letters, 26 (1990) 89–94
Elsevier

IMLET 01479

Inhibition of murine cytotoxic T cell responses by progesterone

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(Received 15 May 1990; accepted 27 June 1990)

1. Summary

In the present study we evaluated the effect of sex hormone on the generation of murine cytotoxic T cell responses. We show that the in vitro CTL response was strongly inhibited by progesterone but not by E_1, E_2 or testosterone. Our experiments attempting to understand the mechanism of the hormone action on CTL development have revealed that the ability of the cells to generate helper signals was not affected. This was demonstrated by the fact that neither IL-2 production nor IL-2 receptor expression was altered by the hormone. Rather, it appears that the capacity of the cells to respond to the signals and to become cytotoxic was modified. Furthermore, we show that the hormone mediated an inhibition of CTL development in thymocyte cultures externally supplemented with all the required helper factors. These results strongly suggest that progesterone has a direct effect on the differentiation of cytotoxic effector cells.

2. Introduction

In spite of the considerable data accumulating in the literature regarding the role played by the gonadal steroid hormones in regulating immune responses [1–3], controversy still exists as to the immune functions affected and the specificity of the regulatory hormones. Furthermore, very little is known, as yet, about the mechanism of action of the hormones on immune reactivities.

Analysis of the effect of gonadal steroid hormones on the different lymphoid cell populations and on the functions of the immune system in the mouse as well as in the human have revealed a variety of effects that are often contradictory. In the mouse, a direct inhibitory activity of 17β-estradiol (E_2) on natural killer (NK) cells was observed [4, 5]. Furthermore, O'Hearn [6] reported that E_2 and progesterone, in concentrations that block DNA synthesis and CTL development, bring about a decreased generation of suppressor cells. In vivo administration of E_2 and testosterone causes depletion of Lyt2$^+$ cells in the thymus whereas only E_2 affects the periphery in a similar manner [7]. According to this report, E_2 mediates reduction in IL-2 production/cell and in suppression activity of splenocytes. These results are in disagreement with those reported by Weinstein et al. [8] showing a superior MLR and antigen presenting ability by female splenocytes and lymph node cells over those of males. This inferior capacity of male lymphocytes could be reversed by castration of the mice and the superiority of the female cell by administration of testosterone.

In the human the picture is even more complex. An E_2-induced enhanced B cell maturation via inhibition of suppressor T cells was reported by Paavonen [9], whereas a progesterone-induced increase in the generation of con A-induced suppressor cells was shown by Holdstock [10].

The confusion expressed in the literature regarding the sex hormone effect on the immune system

Abbreviations: CTL, cytotoxic T lymphocyte(s); IL-2, interleukin-2; NK, natural killer; con A, concanavalin A; PMA, phorbol 12-myristate 13-acetate; E_1, estrone; E_2, 17β-estradiol.

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may be due to the fact that a wide range of hormone concentrations have been tested in different immunological systems, each of which is mediated by different signals [1]. As to T cell activation, most of the studies analysed the effect of the hormones on proliferation, i.e., thymidine incorporation [1], which is the end result of the activation process, and not on each one of the activation steps separately. In the present study we investigated the effect of β-estradiol, progesterone, estrone and testosterone on each one of the major steps of T cell activation and differentiation pathway. We put special emphasis on distinguishing between the hormone effect on the capacity of T cells to provide helper signals for their activation by producing helper factors, and between their ability to respond to these signals by generating receptors and by differentiating into cytotoxic T cells.

3. Materials and Methods

3.1. *Mice*

Mice were bred in the animal facility of the German Cancer Research Center and used at 4–9 weeks of age.

3.2. *Culture medium*

The culture medium used was RPMI-1640 (Gibco; powdered medium) with L-glutamine (2×10^{-3} M) and supplemented with 10% heat-inactivated fetal calf serum (FCS), Hepes (0.02 M), 2-mercaptoethanol (4×10^{-5} M), and gentamicin (50 μg/ml).

3.3. *Hormones*

Solutions (10^{-3} M) of the following hormones were prepared in ethanol: β-estradiol, estrone, testosterone, progesterone (all from Sigma). Further dilutions were prepared in culture medium.

3.4. *Lymphokine preparations*

Con A- (concanavalin-A; Pharmacia, Uppsala, Sweden) induced supernatants were prepared by conventional methods. Single cell suspensions from mouse spleens were adjusted to 10^7 cells/ml in culture medium and incubated for 24 h with con A 5 μg/ml at 37 °C in a humidified 5% CO_2 atmosphere. All preparations were stored at 4 °C.

3.5. *Preparation of IL-2*

Partially purified IL-2 was prepared as described in detail elsewhere [11]. Briefly, EL-4 thymoma cells were activated with phorbol myristate acetate (PMA). IL-2 from the cell-free supernatant was further purified by ammonium sulfate precipitation and consecutive chromatography on phenyl-Sepharose (Pharmacia) and Sephadex G-100.

3.6. *Assay for IL-2 activity*

IL-2 in supernatants was measured by the ability to support growth of the IL-2-dependent cell line W-2 [11]. W-2 cell proliferation was assessed following 20 h of incubation using a 4-h [^{3}H]thymidine pulse (1 μCi of [methyl-^{3}H]thymidine, [^{3}H]TdR; specific activity 50 Ci/mmol; Amersham International Ltd., Amersham).

3.7. *T cell cytotoxicity assays*

Splenic or thymic responder cells at indicated numbers were co-cultured in a final volume of 5 ml or 0.16 ml medium with or without IL-2 and/or lymphokine preparations, with X-irradiated (1500 rad) allogeneic or glutaraldehyde-fixed, TNP-haptenated syngeneic spleen cells as stimulators. Cytotoxic activity was measured on day 5 in a 4-h ^{51}Cr-release assay using 5×10^3 of either unmodified or TNP-modified tumor target cells: L929 mouse fibrosarcoma or P815 mouse mastocytoma. The data are presented as specific ^{51}Cr release calculated as follows:

$$\frac{\text{(experimental release} - \text{spontaneous release)}}{\text{(total release} - \text{spontaneous release)}} \times 100$$

Every effector to target cell ratio was tested in triplicate. The standard error of the mean was always less than 2.5%.

3.8. *Proliferation assays*

Spleen cells (3×10^5) were cultured with 3×10^5 X-irradiated (1500 rad) allogeneic spleen cells as stimu-

lators or with con A (4 μg/ml) in 0.2-ml cultures for 48 h. [^{3}H]TdR uptake was measured following a 16-h pulse. The results are expressed as the mean cpm $\pm$ SD of triplicate cultures.

3.9. *IL-2 receptor determination*

Monoclonal antibodies (AMT 13) from a rat hybridoma cell line directed against a murine IL-2 receptor were a kind gift from T. Diamantstein [12]. Glutaraldehyde-fixed sheep erythrocytes (5×10^6) were added to the triplicate cultures of 4×10^5 thymocytes and the cells were kept on ice for 30 min with or without 100 μl culture supernatant containing AMT 13 antibodies. After another incubation for 60 min on ice with ^{125}I-labeled F(ab')$_2$ fragments of a sheep anti-rat IgG (100 μl of a 1:10 diluted stock with specific activity of 12 μCi/μg, Amersham), the cells were washed, specific binding was determined and expressed as the mean cpm $\pm$ SD of triplicate cultures.

4. Results

The effect of sex hormones on allogeneic stimulation of murine spleen cells was investigated. Coculturing of spleen cells from female BALB/c mice with X-irradiated splenic stimulator cells from female C3H mice for 5 days resulted in a cytolytic T cell response against L929 target cells. The presence of progesterone (1.25 μM) strongly inhibited the generation of cytotoxic activity, estradiol and estrone exhibited a lesser effect and testosterone was almost ineffective (Fig. 1). Similar results were obtained in C3H anti-BALB/c cultures (data not shown).

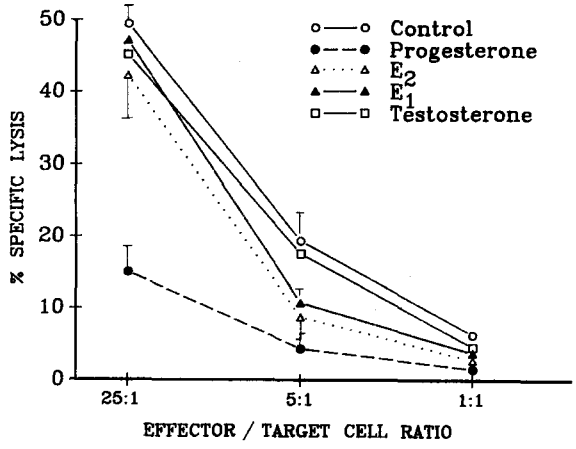

Fig. 1. The effect of steroid hormones on CTL development in allogeneic spleen cell cultures. Spleen cells (2×10^7) of female BALB/c mice were cultured with X-irradiated spleen cells (5×10^6) from female C3H mice in 5 ml medium without or with 10^{-6} M of the indicated hormone. Following 5 days in culture, cells were admixed with ^{51}Cr-labeled L929 tumor cells (5×10^3) in triplicate for a 4-h ^{51}Cr release assay.

The possibility that the reduced response seen in the progesterone and estradiol containing cultures was due to cytolysis of the effector cells by the hormones was ruled out. No decrease, but rather a slight increase, in the viability of cells stimulated in the presence of hormones, as compared to those stimulated with con A alone, was observed (Table 1).

The lower cytotoxicity observed could be the result of intervention by the hormone in different stages of T cell activation. (a) The production of helper factors such as IL-2. (b) The generation of receptors to IL-2. (c) The development of suppressor cells rather than cytotoxic cells.

In order to understand the mechanism(s) responsible for the altered T cell response described above, we present here a study in which we investigate the effect of the hormones on each of the above steps: IL-2 production did not seem to be affected by the hormones. This was tested on splenocytes stimulated with con A and on EL4 cells stimulated with phorbol myristate acetate (PMA). Hormone concentration ranged from 10 μM to 1 μM (data not shown).

TABLE 1

The effect of hormones on the viability of allogeneically stimulated splenocytes.

Hormone concentration in cultures (μM)		Cell number $\times10^{-6}$	% Recovery
None		8.4	100
Progesterone	2.5	8.0	95
	1.25	12.0	142
Estradiol	2.5	8.4	100
	1.25	10.0	119
Estrone	2.5	10.0	119
	1.25	8.4	100
Testosterone	2.5	8.4	100
	1.25	7.6	90

Spleen cells (2×10^7) of female BALB/c mice were cultured with 5×10^6 irradiated spleen cells from female C3H mice in a 5-ml volume. Five days later the cells were washed and viability was determined by trypan blue dye exclusion.

TABLE 2

Effect of steroid hormones on the induction of IL-2 receptor expression on thymocytes.

Hormone added[a]	AMT 13 binding[b] (cpm × 10^{-3})
None	73.1 ± 8.5
Progesterone	84.9 ± 12.3
Estradiol	82.6 ± 2.8
Estrone	70.4 ± 10.2
Testosterone	76.3 ± 4
Fresh thymocytes	6.9 ± 1.4

[a] Cultures of 5×10^6 thymocytes from female C_3H mice were supplemented with 300 μl con A-lymphokine and con A (5 μg/ml) and 1.25 μM of the indicated hormone in a total volume of 1 ml. [b] Specific binding of the anti-IL-2 receptor antibodies (AMT 13) was determined on 4×10^5 cells/sample after 72 h.

The level of IL-2 receptor expression, as well, was not affected by progesterone and estradiol (Table 2). To determine this, thymocytes were stimulated for 72 h with supernatants of splenocytes stimulated with con A in conjunction with con A. We have used this system since thymocytes cannot produce IL-2 but can generate receptors to the factor when external help is provided.

In order to distinguish between the effect of the hormones on the generation of helper signals and between their effect on the ability of the cells to respond to these signals we have tested the effect of the different hormones on the generation of allogeneic thymic responders. Such cultures are dependent upon exogenous IL-2 in order to generate CTL. Thus, T cell malfunction, if detected, could be solely related to the T cell effector arm. Indeed, the development of BALB/c anti-C3H (Table 3) as well as the C3H anti-BALB/c (data not shown) thymocyte response in the presence of excess amount of IL-2 was clearly inhibited by progesterone. In this experimental system the stimulator cells underwent X-irradiation (1500 rad) thus maintaining their capacity to provide helper factors, other than IL-2, that are needed for the development of alloresponse. When using metabolically inactive stimulator cells these helper factors have to be added into the thymocyte cultures in order to get alloresponse [11]. But also in such lymphokine-driven thymocyte cultures, the generation of syngeneic CTL responses was effectively inhibited by progesterone (Table 4). Our results strongly suggest that the hormone does not affect the production of helper signals but probably alters the ability of the cells to respond to these signals.

To address this question we have assessed the capacity of splenocytes to proliferate in response to allogeneic or mitogenic stimuli. As seen in Tables 5

TABLE 3

Effect of steroid hormones on CTL development in allogeneic thymocyte microcultures.

Hormone concentration in cultures[a] (μM)		% Specific lysis BALB/c anti-C_3H[b]
None		53.6 ± 10.3
Progesterone	2.5	23.6 ± 7.1
	1.25	32.9 ± 5.9
Estradiol	2.5	42.1 ± 6.1
	1.25	50.6 ± 5.9
Estrone	2.5	48.1 ± 10.6
	1.25	47.9 ± 1.4
Testosterone	2.5	51.8 ± 7.1
	1.25	41.4 ± 7.8

[a] Thymocytes (10^5) of female BALB/c mice were cultured with 3×10^5 X-irradiated spleen cells from female C_3H mice in 0.16 ml volume. All cultures contained partially purified IL-2 (40 U/ml). [b] After 5 days of culture, 5×10^3 labeled L929 tumor cells were added for 4-h ^{51}Cr-release assay.

TABLE 4

Effect of steroid hormones on CTL development in syngeneic thymocyte microcultures.

Hormone concentration in cultures[a] (μM)		% Specific lysis BALB/c anti TNP C_3H[b]
None		27.1 ± 4.8
Progesterone	2.5	6.0 ± 0.8
	1.25	16.6 ± 4.6
Estradiol	2.5	17.7 ± 2.5
	1.25	26.7 ± 6.0
Estrone	2.5	17.4 ± 3.3
	1.25	25.0 ± 4.9
Testosterone	2.5	21.2 ± 4.2
	1.25	30.2 ± 4.5

[a] Thymocytes (10^5) of female BALB/c mice were cultured with 3×10^5 TNP-modified glutaraldehyde-fixed spleen cells from male C_3H mice in 0.16 ml volume. All cultures contained 60 μl of supernatant of con A-stimulated splenocytes and partially purified IL-2 (40 U/ml). [b] After 5 days of culture, 5×10^3 labeled TNP-modified L929 cells were added for 4-h ^{51}Cr-release assay.

TABLE 5

Effect of steroid hormones on the proliferation of allogeneically stimulated splenocytes.

Hormone concentrations in culture[a] (μM)	cpm $\times 10^{-3}$[b]		
	2.5	1.25	0.6
None	13.1 ± 1.9		
Progesterone	4.2 ± 0.7	4.4 ± 0.8	6.2 ± 1.1
Estradiol	9.2 ± 1.2	15.6 ± 0.7	13.6 ± 0.8
Estrone	8.6 ± 0.9	8.5 ± 0.7	13.3 ± 0.9
Testosterone	10.5 ± 2.2	13.3 ± 0.2	14.1 ± 1.6

[a] Spleen cells (3×10^5) of female BALB/c mice were cultured with 3×10^5 X-irradiated (1500 rad) C_3H splenocytes for 48 h. [b] [^{3}H]Thymidine incorporation was assessed following a 16-h pulse and is expressed as cpm of triplicate cultures.

and 6, a concentration of as low as 0.5 μM progesterone strongly inhibited the proliferative capacity of BALB/c cells stimulated either with C3H spleen cells or with con A. The C3H splenocyte activation either by con A or by lymphokine containing supernatants, as well as the DBA anti-C3H stimulation were similarly inhibited by progesterone (data not shown). No effect was detected by estradiol, estrone, or testosterone. These results indeed indicate that the hormone interferes with the generation of effector cells by a mechanism not yet understood.

5. Discussion

The data presented in this article demonstrate a strong inhibitory effect of progesterone, but not of E_1, E_2 or testosterone, on the generation of murine CTL responses. These results are consistent with those reported by others (for review, see ref. 1) showing a marked progesterone-mediated inhibition of proliferation, of rat as well as of human lymphocytes, following mixed lymphocyte reaction or mitogenic stimulation. In addition, graft rejection experiments in mice and rats have shown that the hormone can reduce the in vivo inflammatory processes.

The concentration of the hormone used in our experiments was around 10^{-6} M which is very similar to those present in the circulation during pregnancy [13–15]. Since elevated levels of circulating hormone are present during pregnancy in many animal models as well as in humans [1, 13–15], it is suggested that progesterone produced by the placenta and/or ovaries during pregnancy may inhibit T lymphocyte-mediated responses involved in tissue rejection. In our study in the human, using peripheral blood mononuclear cells from post-menopausal women [16], we could not show any effect of progesterone on all the immunological functions tested. If the hormone-mediated modulation of the immune response indeed plays a role in pregnancy, it is possible that in the post-menopausal age the lymphocytes lose their sensitivity to the hormones.

Our experiments attempting to understand the mechanism of the hormone action on CTL development have revealed that the ability of the cells to generate helper signals was not affected. This was demonstrated by the fact that neither IL-2 production nor IL-2 receptor expression was altered by the hormone. Rather, it appears that the capacity of the

TABLE 6

Effect of steroid hormones on the proliferation of con A-stimulated splenocytes.

Hormone concentrations in culture[a] (μM)	cpm $\times 10^{-3}$[b]		
	2.5	1.25	0.6
None	224.4 ± 19.0		
Progesterone	143.4 ± 11.9	128.1 ± 20.7	128.1 ± 19.3
Estradiol	210.3 ± 15.1	232.3 ± 10.1	207.7 ± 11.7
Estrone	213.2 ± 5.4	219.4 ± 10.4	212.3 ± 18.7
Testosterone	176.0 ± 9.4	182.6 ± 26.7	209.0 ± 9.0

[a] Splenocytes (3×10^5) of female BALB/c mice were cultured with 4 μg/ml con A for 48 h. [b] [^{3}H]Thymidine incorporation was assessed following a 16-h pulse and is expressed as cpm of triplicate cultures.

cells to respond to the signals and to become cytotoxic was modified. Furthermore, the data demonstrating an inhibition of CTL development in thymocyte cultures supplemented with all the required [11] helper factors (Table 4), emphasize the direct effect of progesterone on the differentiation of cytotoxic effector cells.

Several investigators suggest that progesterone affects the immune response by modulating the generation and/or the activity of suppressor cells. Indeed, significant increase in suppressor cell activity was observed in a mitogen-induced suppressor T cell system in the human [10]. On the other hand, a direct inhibition of suppressor cell function by progesterone was also reported [6]. The discrepancy between the two observations can be due to differences between the experimental systems used. However, in our system, it does not seem plausible that suppressor cells can play a major role in our 5-day thymocyte cultures, even though this possibility has not been experimentally excluded.

E_2 was found to be almost ineffective in our system in spite of the relatively high concentrations used. The direct in vitro effect of E_2 on blastogenesis is rather controversial. Many investigators (for review, see ref. 1) including ourselves, in our study in the human [16], confirm the result presented in this study. However, it seems that in those cases where an effect could be shown it was due to the high concentrations used [1].

It should be noted that there are convincing data as to the effect of E_2 on other immunological functions, namely, on pokeweed mitogen-induced human B cell differentiation [9, 17], on antibody formation [18], on autoantibody production by $CD5^+$ B cells [3, 19], etc. The hormone mechanism of action is not yet understood.

As mentioned above, progesterone did not seem to modulate IL-2 receptor expression (Table 2), however, we cannot rule out the possibility that the hormone alters the receptor or affects differentially the low affinity receptors [20].

Even though the molecular mechanisms of the progesterone-induced inhibition of proliferation of T cells and of CTL development have not been completely elucidated, the observations of this report support the potential importance of progesterone as an immunomodulator.

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