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Complement C3 synthesis, peroxidase activity and eosinophil chemotaxis in the rat uterus: effect of estradiol and testosterone

Maria C. Leiva¹, C. Richard Lyttle¹ and Peter H. Jellinck²

¹ Department of Obstetrics and Gynecology, Division of Reproductive Biology, University of Pennsylvania, Philadelphia, PA 19104, U.S.A., and ² Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada

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Summary

Treatment of immature rats with estradiol (E₂) produced a large increase in uterine peroxidase activity which was accompanied by an increase in eosinophil chemotactic factor (ECF-U). The synthesis of complement C3 was also induced in the uterus and the amount of this 180 kDa protein was determined both by immunoprecipitation and after separation by polyacrylamide gel electrophoresis. Testosterone (T) did not produce an increase in any of these parameters although it antagonized the estrogen-induced increase in uterine peroxidase activity and these effects were more pronounced in estrogen-primed animals. This antagonism was prevented by the antiandrogen, flutamide. Testosterone showed little effect on eosinophil chemotactic activity and did not inhibit the E₂-stimulated synthesis of C3. The results with T were supported by the lack of any significant effect by flutamide which antagonizes receptor-mediated androgenic events. These findings are discussed in relation to the action of other types of hormonal steroids (progesterone, dexamethasone) in inhibiting these estrogen-induced molecular changes in the rat uterus and contribute to our understanding of steroid–steroid interaction and the regulation of uterine function.

Introduction

Treatment of immature female rats with estradiol (E₂) causes many molecular changes in the uterus which can be modulated by progestins, corticosteroids, androgens and other hormones. Most of these responses such as those involving the progesterone receptor (Milgrom et al., 1970), creatine phosphokinase (Kaye, 1970), insulin-like

growth factor (Murphy and Friesen, 1988), various secreted proteins (Komm et al., 1986; Kuivanen and DeSombre, 1986; Takeda et al., 1988; Kuivanen et al., 1989) and actin and collagen mRNA (Hsu and Frankel, 1987; Komm et al., 1987) result from the stimulation of one or more uterine cell types. However, the estrogen-induced increase in uterine peroxidase activity (Lyttle and Jellinck, 1972; Lyttle and DeSombre, 1977) is not derived from resident uterine cells but results primarily from infiltration of eosinophils into the uterus (Ross and Klebanoff, 1966; Tchernitchin, 1974; King et al., 1981). This increase in peroxidase activity produced by treatment with estrogen

Address for correspondence: Dr. P.H. Jellinck, Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada.

can be prevented by the co-administration of actinomycin D, an inhibitor of RNA synthesis (Lyttle and DeSombre, 1977), by emetine, an inhibitor of protein synthesis (Keeping and Lyttle, 1982a), by progesterone which decreases the level of estrogen receptors (Keeping and Lyttle, 1982b), by antiestrogens (McNabb and Jellinck, 1976; Keeping and Lyttle, 1982b), by corticosteroids (DeSombre and Lyttle, 1979), and by androgens (Jellinck et al., 1983). The effect of the androgen can be reversed by flutamide, an anti-androgen known to act through the androgen receptor (Jellinck and Newcombe, 1983).

These results, coupled with the findings that uterine peroxidase activity is due to an influx of eosinophils rich in this enzyme (King et al., 1981; Keeping and Lyttle, 1984) suggest that E_2 and testosterone (T) acting via their own individual receptors may increase the production of factors which stimulate or inhibit the infiltration of eosinophils into the uterus. Such an estrogen-induced chemotactic factor (ECF-U) which is heat labile, pronase sensitive and with a mass of approximately 18 kDa has now been partially purified (Lee et al., 1989) but the nature of possible inhibitory factors produced by steroid hormones has not been identified.

We therefore undertook to determine whether the inhibition of uterine peroxidase activity by T could be correlated with an inhibition of the chemotactic factor and also whether this androgen antagonized other estrogen-regulated uterine responses such as the induction of complement component C3. This 180 kDa protein (C3) was chosen because derivatives of this complement component have been shown to possess chemotactic activity towards neutrophils and eosinophils (Larsen and Henson, 1983).

Materials and methods

Reagents

Chemicals and methionine-free minimum essential medium (MEM) were purchased from Sigma (St. Louis, MO, U.S.A.); calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS) was purchased from Gibco (Grand Island, NY, U.S.A.). Flutamide (4'-nitro-3'-trifluoromethylisobutyranilide) was a generous gift of

the Schering Corporation (Dr. A. Watnick). All other chemicals were the purest available commercially.

Animals

Immature female Sprague-Dawley rats (16–17 days old) from Charles River, La Prairie, Quebec, Canada, were divided into groups of 6–7 at random and housed in a temperature-controlled room (22°C) with a 14:10 light:dark cycle. Primed animals received 3 μ g of estradiol (E_2) in sesame oil containing 10% ethanol (0.2 ml) s.c. on the day of arrival (day 1). On day 8, two groups of rats were given 0.5 mg testosterone (T) or T + 1 mg flutamide (F) at 09.00 h and then E_2 (1 μ g) with another dose of T or T + F at 16.00 h. A third group received E_2 . The first dose of F preceded T by 5 min, but was mixed with T for the later injections. On day 9, the administration of E_2 , E_2 + T or E_2 + T + F was repeated at 14.00 h and the animals were killed by cervical dislocation at 09.00 h on day 10 at age 26–27 days. In some experiments T was replaced by its longer acting propionate (Tp).

Determination of uterine peroxidase activity

The tissue was dissected free of adhering fat, blotted and weighed. It was cut into small pieces and homogenized in the appropriate volume of 10 mM Tris-HCl (pH 7.4) to give a 2.5% (w/v) homogenate using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at $40,000 \times g$ for 30 min at 4°C and the supernatant fraction used for the chemotactic assay (see below). The pellet was resuspended by homogenizing in the same volume of 10 mM Tris-HCl containing 0.5 M $CaCl_2$ and centrifuged again at $40,000 \times g$ for 30 min to obtain a supernatant containing most of the peroxidase activity in the uterus.

Peroxidase activity was determined using guaiacol as substrate (Himmelhoch et al., 1967). The reaction mixture (3 ml) contained guaiacol (13 mM) and H_2O_2 (0.33 mM) in 10 mM Tris-HCl (pH 7.4) and 1.0 ml of the sample. The linear increase in absorbance at 470 nm resulting from the oxidation of guaiacol was followed in a recording spectrophotometer and expressed as absorbance units/min/g tissue.

In vitro incubation

Uteri were rinsed 3 times in minimal essential medium (MEM; Sigma, St. Louis, MO, U.S.A.) minus methionine and incubated for 12 h at 37°C, 5% CO₂ in MEM minus methionine containing penicillin (100 µg/ml), streptomycin (1 µg/ml), and 25 µCi/ml [³⁵S]methionine (1200 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.). The incubation media were collected after 6 h and the cellular debris removed by centrifugation. Radioactivity incorporated into protein was determined by trichloroacetic acid (TCA) precipitation and the proteins analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Komm et al., 1986). Fluorograms were prepared and developed after a 24–48 h exposure.

Immunoprecipitation of complement C3

15 µl of goat anti-rat C3 antibody (Cappel, Organon Teknika Corp., West Chester, PA, U.S.A.) were added to each 100 µl of sample volume containing a total of 50,000 TCA precipitable counts per minute. The samples were placed on a shaker at 4°C for 24 h after which 50 µl of prepared protein-A (Pansorbin; Cal-Biochem Corp., LaJolla, CA, U.S.A.) were added, incubated for 1 h, and a pellet obtained by centrifugation at 5000 × g for 5 min. The pellet was washed by resuspension followed by centrifugation. The washing was repeated 3 times with Tris-buffered saline (TBS) containing 0.5% Tween 20 and 0.05% SDS, and once with TBS. The washed pellet was resuspended in 100 µl of 2 times final sample buffer without reducing agent, heated at 90°C for 10 min, centrifuged, and 10 µl aliquots of the supernatant counted in a scintillation counter. Newly synthesized and secreted C3 was quantitated by the total counts per minute recovered with the anti-C3 antibody.

Preparation of chemotactic cells

Clone 15 HL-60 cells can differentiate into eosinophil-like cells (HL-60/Eos) (Fischkoff et al., 1984). These differentiated HL-60/Eos respond to a variety of eosinophil chemotactic factors in a physiological manner, a response not seen in undifferentiated HL-60 cells (Howe et al., 1990). Clone 15 cells were cultured in RPMI-1640

with 10% heat-inactivated fetal calf serum (HyClone) in a humidified atmosphere of 5% CO₂ at 37°C, pH 7.4. One week before chemotactic experiments, the medium was made 0.3 mM in butyric acid, and cells were cultured without changing the medium for 7 days. These differentiated HL-60 cells were then collected and washed as described previously (Howe et al., 1990). The washed cells were suspended in PBS at 1.0–1.5 × 10⁶ viable HL-60 Eos/ml for chemotaxis.

Chemotaxis assay

The assays for chemotaxis were performed as described previously (Lee et al., 1989), using a 48-well blind well chamber (Neuro Probe, Cabin John, MD, U.S.A.) (Falk et al., 1980) and 5-µm polycarbonate membrane (Harvath and Leonard, 1980) (Nucleopore, Cutter's Mills, CA, U.S.A.). Briefly, 50 µl of cell suspension containing 50,000–75,000 viable HL-60/Eos were added to each upper well of the chemotaxis chamber and incubated for 60 min at 37°C in humidified 5% CO₂. The chamber was disassembled, and the

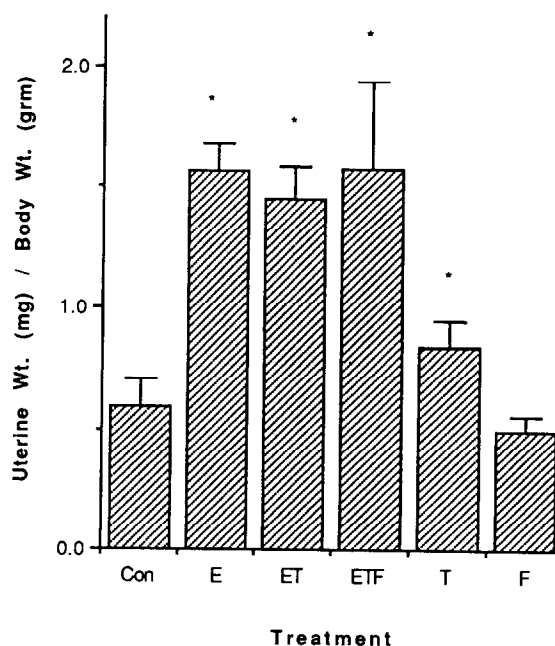


Fig. 1. Effect of estradiol (E), testosterone (T) or flutamide (F) on uterine/body weight ratio. Rats were injected s.c. with E (1 µg) or E+T (0.5 mg) or E+T+F (1 mg) on two successive days and the animals were killed on day 3. * $P < 0.01$ compared with control.

membrane removed, fixed in methanol, and stained with Wright-Giemsa (Hemacolor, EMI Diagnostics, Gibbstown, NJ, U.S.A.). Chemotaxis was assessed by counting the number of cells that had migrated through the membrane and were fixed to its surface. Each extract was run in 3–12 wells, and the results were averaged to obtain a value for the individual animal; counts (expressed as cells per well) were compared to those of uterine extracts from control animals.

Results

The effect of treatment of unprimed immature rats with E_2 ($2 \times 1 \mu\text{g}$) at 24 h intervals (killed on day 3) on uterine/body weight ratio is shown in Fig. 1. The estrogen caused an almost 3-fold increase which was not influenced by the co-administration of either testosterone (T) or T + flutamide (F). Injection of T alone ($2 \times 0.5 \text{ mg}$) produced some increase in this ratio but F ($2 \times 1 \text{ mg}$) was without effect.

E_2 produced a large increase in the 180 kDa protein, now known to be complement component C3 (DeSombre et al., 1989; Sundstrom et al., 1989) but T did not induce this protein nor did T or its propionate (Tp) reverse the effect of E_2 (Fig. 2). Complement component C3 was measured by immunoprecipitation (Fig. 2A) or densitometry after fluorography (Fig. 2B) of the products separated by SDS-PAGE flutamide, as expected, did not influence the production of C3. The fluorogram of the E_2 -induced C3 is shown in Fig. 3. Neither T nor F influenced the formation of this protein or of its mRNA as determined by Northern analysis using a specific radioactive cDNA probe (not shown).

Treatment with T did not antagonize the increase in eosinophil chemotaxis produced by uterine extracts from immature rats injected with E_2 ; F, given together with T, also showed no effect (Fig. 4).

These results are in sharp contrast to the effect of T and F on the E_2 -induced increase in peroxidase activity (Fig. 5). T and also its longer

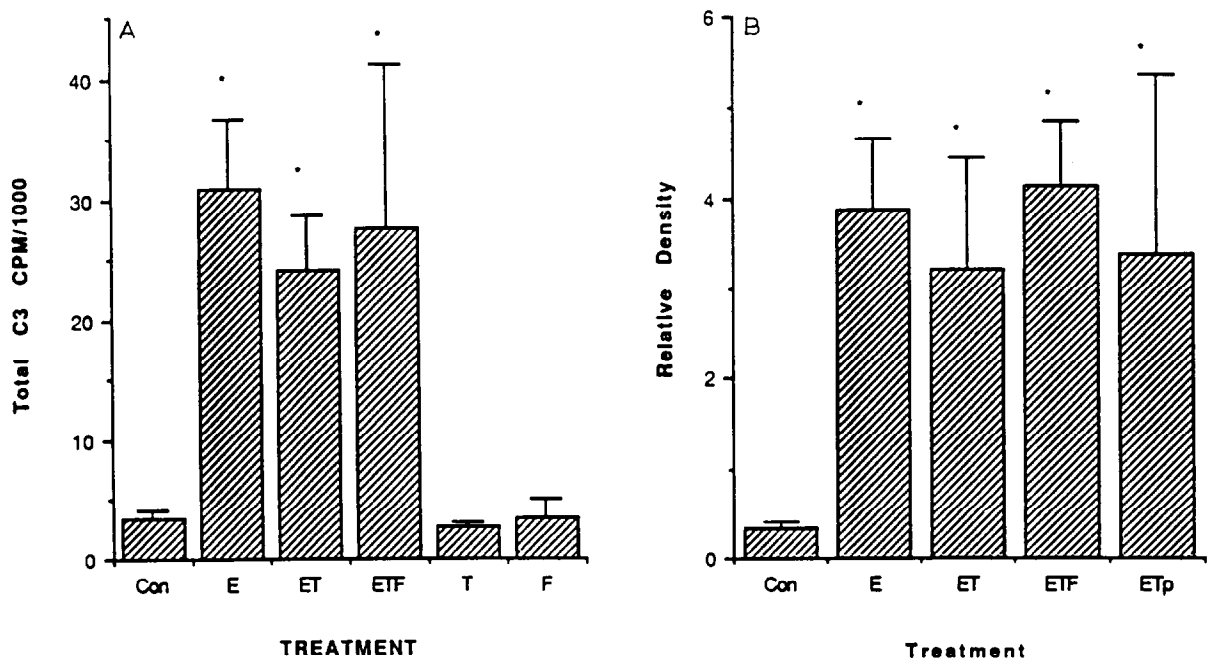


Fig. 2. Effect of estradiol (E), testosterone (T), testosterone propionate (Tp) or flutamide (F) on the induction of complement C3. The rats were treated as in Fig. 1 and uteri incubated with [^{35}S]methionine. C3 was determined by immunoprecipitation (A) or by densitometry after separation of the secreted products by SDS-PAGE (B) as described in Materials and methods. Tp was administered at 0.5 mg/rat. * $P < 0.01$ compared with control.

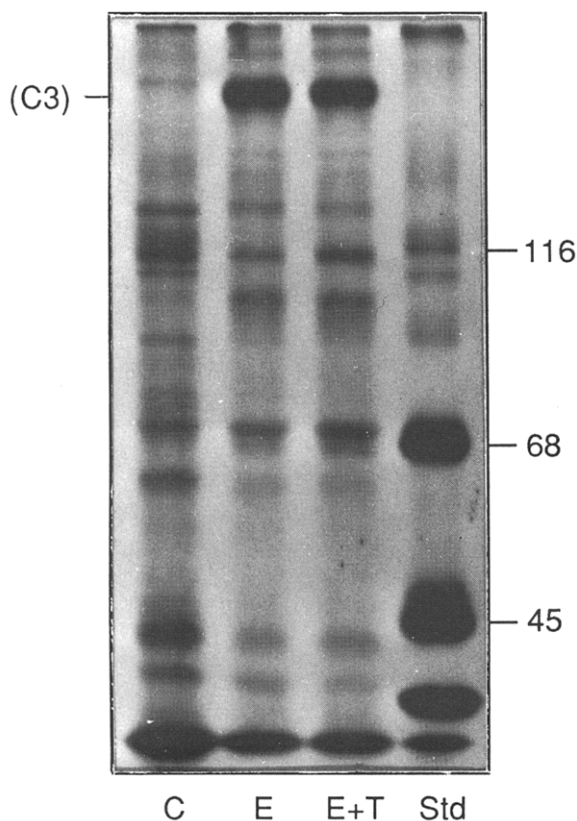


Fig. 3. Comparison of ^{35}S -labeled medium proteins from control, and estradiol-stimulated rat uteri after treatment with testosterone or flutamide. Each lane was loaded with an equal amount of TCA-precipitable radioactive proteins obtained from the medium after incubation for 6 h. Proteins were separated on a 7.5% SDS-polyacrylamide gel followed by fluorography.

acting propionate (Tp) produced a marked decrease in the activity of this enzyme which was reversed by F. These effects were accentuated by using animals primed 7 days earlier with E_2 (3 μg).

Discussion

It is now well established that most of the peroxidase that is present in the rat uterus after *in vivo* stimulation with estrogen is of eosinophilic origin (King et al., 1983) and that a directed migration of these cells along a concentration gradient may be the result of chemotactic factors. Recently, it was demonstrated that a protein of molecular mass 18 kDa (ECF-U), produced in the

rat uterus in response to E_2 also had strong eosinophil chemotactic properties (Lee et al., 1989).

Many of the changes occurring in the uterus following E_2 stimulation resemble an inflammatory response and, therefore, it has been suggested the eosinophils provide the enzymes needed for remodelling of the endometrial stroma (Ross and Klebanoff, 1966) and that they may play a role in preparing the endometrium for blastocyst implantation. Eosinophil peroxidase may be involved in the regulation of estrogen binding and metabolism (Lyttle and Jellinck, 1972; Lyttle et al., 1984).

The estrogen-induced increase in peroxidase activity can be prevented by concurrent treatment with a number of agents including antiestrogens, progesterone, corticosteroids as well as androgens (see Introduction). However, the action of androgens on the synthesis of the chemotactic

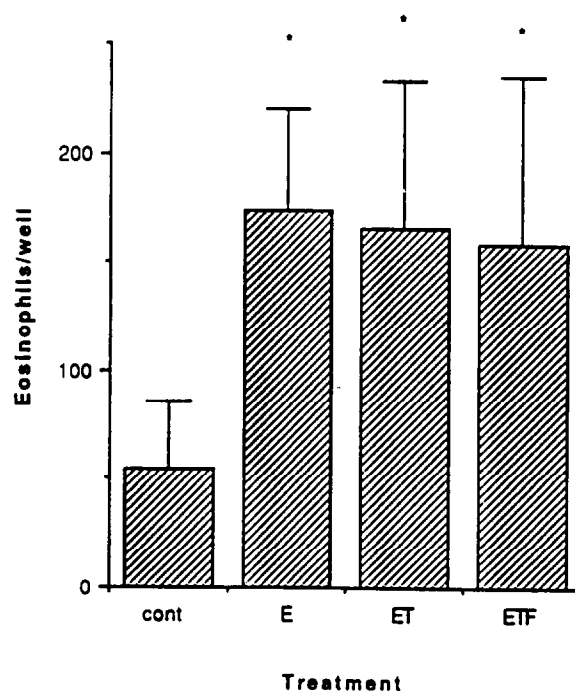


Fig. 4. Effect of estradiol (E), testosterone (T) or flutamide (F) on the induction of uterine chemotactic factor ECF-U. Rats were injected s.c. with E (1 μg), or E+T (0.5 mg), or E+T+F (1 mg) on two successive days and uterine extracts prepared 24 h later for assay of chemotactic activity as described in Materials and methods. * $P \leq 0.01$ when compared to control.

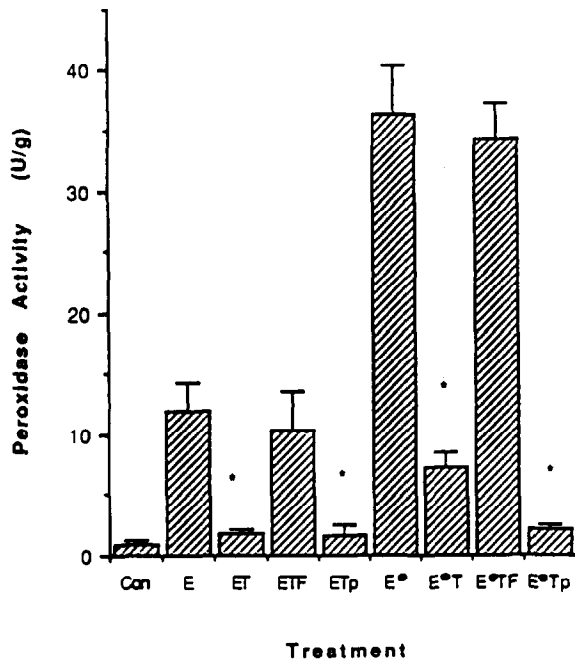


Fig. 5. Effect of estradiol (E), testosterone (T), testosterone propionate (Tp) or flutamide (F) on the induction of uterine peroxidase activity. Treatment was as in Fig. 1 but four groups of rats were 'primed' with estradiol (●) 7 days before further injection of the test compounds. Each group had a minimum of nine animals. * $P \leq 0.01$ compared with estradiol stimulation.

factor or of the C3 component of complement has not been determined.

It had been shown earlier that three daily injections of testosterone (0.5 mg) given concurrently with E_2 (1 μ g) to immature rats will antagonize the effect of estrogen on peroxidase induction and also that one injection of either T or its propionate (Tp) at 2.5 mg was inhibitory if given at least 8 h before the estrogen (3 μ g) (Jellinck and Newcombe, 1983). In our current experi-

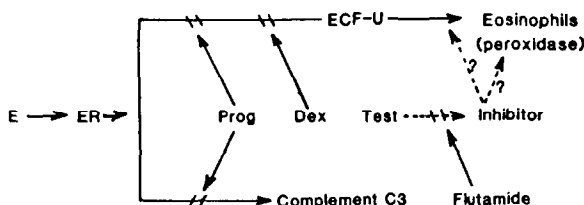


Fig. 6. Outline of the current status of hormonal regulation of uterine infiltration of eosinophils and complement C3 synthesis. R = receptor.

ments, two daily injections of androgen (testosterone or testosterone propionate) were used and peroxidase activity was determined in each individual uterus instead of pooling the tissue as had been done previously. The effect of priming with estrogen one week before treatment, which increases the concentration of androgen receptors (Mobbs and Johnson, 1988), was also assessed and shown to enhance the response to these hormones. However, in contrast to their marked inhibitory effect on uterine peroxidase activity, neither T nor its propionate had any significant influence in antagonizing the effect of E_2 on the induction of complement component C3 or eosinophil chemotactic factor. Synthesis of C3 was measured by immunoprecipitation and by densitometry of the autoradiograms obtained following separation of the 35 S-labeled C3 molecules by SDS-PAGE. In experiments with progesterone or dexamethasone, the suppression of C3 was dramatic (70–90% inhibition) (Wheeler et al., 1987) showing the sensitivity of the response, and any inhibitory activity of T or Tp would have been readily detected in this system. In addition, mRNA levels for C3 were not altered by androgen. Thus, all the evidence points to a lack of effect of T on the increased synthesis of complement component C3 by E_2 .

We had proposed previously (Jellinck and Newcombe, 1983) that T prevents the E_2 -induced increase in uterine peroxidase activity by stimulating the formation of an inhibitor by a process involving the androgen receptor because flutamide reversed this action of T. The inhibitor could either interfere with the synthesis of peroxidase within the eosinophils or prevent the formation of the eosinophil chemotactic factor by the uterus. In our present work very little correlation between the action of androgen on E_2 -induced peroxidase activity in the uterus and chemotaxis of eosinophils was obtained, a divergence which is hard to reconcile in view of the association of this enzyme with these non-resident cells. However, the effect of the putative inhibitor (see Fig. 6) on the chemotactic factor ECF-U was tested on individual cells away from the influence of the whole organism. This inhibitor may have low stability, may require cofactors for activity or may act indirectly by decreasing the synthesis of per-

oxidase within the eosinophils. In addition, it may be formed very early after androgen stimulation and would no longer be present at the time of testing for chemotaxis. A summary of the current status of the hormonal regulation of uterine infiltration by eosinophils is outlined in Fig. 6.

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References

- Czarnetski, B.M. and Rosenbach, T. (1986) Prostaglandins 31, 851-858.
- DeSombre, E.R. and Lyttle, C.R. (1979) Adv. Exp. Med. Biol. 17, 157-171.
- Falk, W., Goodwin, R.C. and Leonard, E.J. (1980) J. Immunol. Methods 33, 239-247.
- Fischkoff, S.A., Pollak, A., Gleich, G.J., Testa, J.R., Misawa, S. and Reber, T.J. (1984) J. Exp. Med. 160, 179-196.
- Harvath, L., Falk, W. and Leonard, E.J. (1980) J. Immunol. Methods 37, 39-45.
- Himmelhoch, S.R., Evans, W.H., Mage, M.G. and Peterson, E.A. (1967) Biochemistry 8, 914-921.
- Howe, R.S., Fischkoff, S.A., Rossi, R.M. and Lyttle, C.R. (1990) Exp. Hematol. 18, 299-303.
- Hsu, C.J. and Frankel, F.R. (1987) J. Biol. Chem. 262, 9594-9600.
- Jellinck, P.H. and Newcombe, A.M. (1983) J. Steroid Biochem. 19, 1713-1717.
- Jellinck, P.H., Alfleck, A. and Newcombe, A.M. (1983) Can. J. Biochem. Cell Biol. 61, 779-783.
- Kaye, A.M. (1970) in Biochemical Actions of Hormones (Litwack, G., ed.), Vol. 5, p. 149, Academic Press, New York.
- Keeping, H.S. and Lyttle, C.R. (1982a) 64th Annual Meeting of the Endocrine Society, San Francisco, CA (Abstract 893).
- Keeping, H.S. and Lyttle, C.R. (1982b) Endocrinology 111, 2046-2054.
- Keeping, H.S. and Lyttle, C.R. (1984) Biochim. Biophys. Acta 802, 399-406.
- King, W.J., Allen, T.C. and DeSombre, E.R. (1981) Biol. Reprod. 25, 859-867.
- Komm, B.S., Rusling, D.J. and Lyttle, C.R. (1986) Endocrinology 188, 2411-2416.
- Komm, B.S., Frankel, F.R., Myers, J.C. and Lyttle, C.R. (1987) Endocrinology 120, 1403-1410.
- Kuivainen, P.C. and DeSombre, E.R. (1985) J. Steroid Biochem. 22, 439-451.
- Kuivainen, P.C., Capulong, R.B., Harkin, R.N. and DeSombre, E.R. (1989) Biochem. Biophys. Res. Commun. 158, 898-905.
- Larsen, G.L. and Henson, M. (1983) Annu. Rev. Immunol. 1, 335-359.
- Lee, Y.H., Howe, R.S., Sha, S.J., Teuscher, C., Sheehan, D.M. and Lyttle, C.R. (1989) Endocrinology 125, 3022-3028.
- Lyttle, C.R. and DeSombre, E.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3162-3166.
- Lyttle, C.R. and Jellinck, P.H. (1972a) Biochem. J. 127, 481-487.
- Lyttle, C.R. and Jellinck, P.H. (1972b) Steroids 20, 89-92.
- Lyttle, C.R., Medlock, K.L. and Sheehan, D.M. (1984) J. Biol. Chem. 259, 2697-2700.
- McNabb, T. and Jellinck, P.H. (1976) Steroids 27, 681-689.
- Milgrom, E., Atger, M. and Beaulieu, E.E. (1970) Steroids 16, 741-754.
- Mobbs, B.E. and Johnson, I.E. (1988) Cancer Res. 48, 3077-3083.
- Murphy, L.J. and Friesen, H.G. (1988) Endocrinology 122, 325-332.
- Ross, R. and Klebanoff, S.J. (1966) J. Exp. Med. 124, 653-659.
- Sundstrom, S.A., Komm, B.S., Ponce-de-Leon, H., Yi, Z., Teuscher, C. and Lyttle, C.R. (1989) J. Biol. Chem. 264, 15941-15947.
- Takeda, A., Takahashi, N. and Shimizu, S. (1988) Endocrinology 122, 105-113.
- Tchernitchin, A., Roerijck, J., Tchernitchin, X., Vandenhende, J. and Galand, P. (1974) Nature 248, 142-143.
- Wheeler, C., Komm, B.S. and Lyttle, C.R. (1987) Endocrinology 120, 919-923.