Int. J. Immunopharmac., Vol. 1, pp. 151-155
Pergamon Press Ltd. 1979. Printed in Great Britain.

0192-0561/79/0401-0151 $02.00/0

SHORT COMMUNICATION

STIMULATING PROPERTIES OF LYNESTRENOL ON NORMAL HUMAN BLOOD T-LYMPHOCYTES AND OTHER LEUCOCYTES

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(*Received in final form* 12 *December* 1978)

Abstract—The influence of lynestrenol, a synthetic progesterone-like drug, has been investigated on normal human blood leucocytes *in vitro*.

Lynestrenol significantly increased the percentage of active T rosettes (a test for T-cell competence) at concentrations of 10^{-1} to 10^{-4} μg/ml (controls: 16.5%; drug at 10^{-1} μg/ml: 23.6%; $P<0.001$). It did not alter the percentage of total T rosettes (a quantitative measure of T cells) and the percentage of EAC rosettes. It increased the non-adherent cells in the leucocyte adherence inhibition test (LAI) over a dose range between 10^{-1} and 10^{-3} μg/ml (controls: 360,000 compared to 517,000 in the presence of 10^{-2} μg/ml; $P<0.001$) suggesting some leucocyte activation. Finally, it also increased the phagocytic properties of monocytes.

These results indicate the lynestrenol acts upon normal human blood T lymphocytes, monocytes and possibly neutrophils. Therefore, lynestrenol appears to possess immunostimulatory properties.

Lynestrenol is a synthetic progesterone-like substance widely used in various gynaecological conditions. In view of postulated relationships between sex hormones and the immune system, the possible effects of lynestrenol upon various, *in vitro*, immunological tests were investigated. This study was based on our previous work which showed that lynestrenol increases both the proliferative responses of human lymphocytes when stimulated by either phytohemagglutinin or the mixed lymphocyte culture reaction, whereas it had no influence upon pokeweed mitogen stimulation (Wybran, Van Bogaert & Govaerts, 1977). We have also shown that lynestrenol alters the growth of transplanted tumors in hamsters by delaying the appearance of malignant tumors and prolonging the survival of tumor-bearing animals (Wybran & Thiry, 1978). In the present *in vitro* study, the influence of lynestrenol on different types of human leucocytes was examined using various tests including lymphocyte rosette assays, leucocyte adherence inhibition tests and measurements of phagocytosis.

EXPERIMENTAL PROCEDURES

Rosette assays

The following rosette tests were performed according to our previously published methods: active T test (TEa) (Wybran, Levin, Spitler & Fudenberg, 1973; Wybran & Govaerts, 1977; Felsburg, Edelman & Gilman, 1976), total T-rosette (TEt) (Felsburg & Edelman, 1977; Wybran, Chantler & Fudenberg, 1973a; Wybran & Govaerts, 1977), non-stained autologous red cells rosettes (TEh) (Lambermont, Wybran & Govaerts, 1977), and erythrocyte-antibody-coated rosettes (EAC) (Wybran & Govaerts, 1977). Briefly, blood mononuclear cells were isolated after centrifugation on a Ficoll-Hypaque layer, washed, and finally adjusted to contain 15×10^6 cells/ml, except for the EAC rosettes where a 30×10^6 cells/ml suspension was used. Aliquots (0.033 ml) of the cell suspension were incubated with 0.1 ml of various dilutions of lynestrenol for 1 h in a waterbath at 37°C. Pure lynestrenol powder was a gift of Organon International, Oss, Holland. It was dissolved in ethanol (3 mg per ml) and further diluted in RPMI 1640 medium containing HEPES buffer (Gibco-Biocult) so that the final concentration of the stock preparation was 30 μg/ml. Lynestrenol was used at concentrations varying between 10^{-1} and 10^{-5} μg/ml. After incubation, the various rosette assays were performed. The tests were always done in duplicate. The results are expressed as the percentage of cells forming rosettes.

Leucocyte adhesion inhibition test (LAI)

Preparation of the tissue extract and the test were performed according to a previously described method (Grosser & Thomson, 1975; Appelboom, Govaerts & Wybran, 1978). The tissue used in the test was a breast tumor extract diluted (1:5) in Medium

199. It was prepared as follows: autopsy breast samples were obtained within 24 h of death. Fatty and fibrous tissues were dissected away and the specimen was homogenized for 10 min in 3 volumes of medium in a Virtis blender. The homogenate was centrifuged at 20,000 *g* for 30 min and the supernatants used in the test were stored at −40°C in 2 ml aliquots (protein concentration, 1 mg/ml). The LAI was performed as follows. Heparinized blood was allowed to settle for 1 h at 37°C in Falcon Plastic Tubes (16×155 mm). The leucocyte-rich fraction was collected and adjusted to 15×10^6 cells/ml of Medium 199. Then, 1.5×10^6 cells were incubated in 0.2 ml of Medium 199 plus 0.1 ml of lynestrenol in medium for 1 h at 37°C.

The final concentrations of the drug in the tests medium with 25 mM HEPES. After the incubation time, 0.1 ml of tissue extract was added to the non-siliconized Glass Kimax tubes (15×125 mm), which were then incubated horizontally for another hour at 37°C, after which time the tubes were placed vertically. An aliquot of nonadherent cells was removed by pipette and counted in a hemocytometer. The tests were always done in duplicate. The results are expressed as the total number of nonadherent cells per mm^3. In another set of experiments, the LAI was performed in the absence of extract with various drug concentrations.

Yeast phagocytosis

Blood mononuclear cells (lymphocytes and monocytes) isolated by centrifugation on a Ficoll-Hypaque gradient were washed and adjusted to a final concentration of 15×10^6 cells/ml. Baker's yeast was washed three times in RPMI 1640 medium containing 25 mM HEPES. The mononuclear cells were incubated with the yeast cells for 45 min at 37°C in a waterbath with or without lynestrenol (10^{-4} to 10^{-1} μg/ml). An aliquot of cells was then examined in a hemocytometer for yeast phagocytosis. A cell (monocyte) was considered phagocytic when it contained at least two yeast completely surrounded by cytoplasm. The tests were done in duplicate and the results are expressed as percentage of phagocytic cells.

Statistical methods

The results are given as mean ± standard error (S.E.M.). A Student *t*-test for paired data was used to compare the control results (no drug) with those obtained with various concentrations of the drug. A value of *P* less than 0.05 was considered significant.

RESULTS

Figure 1 and Table 1 indicate the influence of lynestrenol upon various rosette assays. Compared to the control, a significant increase in TEa percentages was observed (Fig. 1) at lynestrenol concentrations

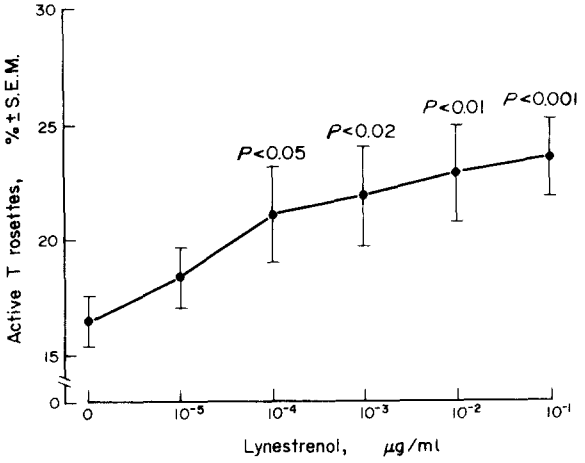

Fig. 1. Effect of various concentrations of lynestrenol on the percentage of active T rosettes (mean of 10 experiments ±S.E.M.)

Table 1. Lynestrenol and rosette assays*

	Lynestrenol concentrations (μg/ml)					
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Total T rosettes	64.1±2.2	63.1±1.9	62.7±2.6	63.1±2.4	65.2±2.4	62.3±2.7
Autologous rosettes	17.6±2.5	N.D.	18.8±3.2	20.3±3.0	20.0±2.9	21.3±1.9†
EAC rosettes	21.8±4.7	N.D.	23.7±5.7	22.0±5.0	22.8±4.5	21.5±4.5

* The results represent the mean ± S.E.M. in percentage of 6 experiments.
† *P* value < 0.02 (relative to controls).
N.D.: not done.

between 10^{-1} and 10^{-4} μg/ml using cells from 10 normal blood donors (5 females and 5 males, 22–59 years old). The greatest increase was observed at 10^{-1} μg/ml. The percentage of autologous red cell rosettes was only increased at the 10^{-1} μg/ml concentration (Table 1). In contrast, both the TEt and EAC percentages remained unchanged at all the drug concentrations studied (Table 1).

The LAI was modified by lynestrenol. There was a significant increase in the number of nonadherent cells at concentrations of 10^{-1}, 10^{-2} and 10^{-3} μg/ml in 8 normal subjects (4 females and 4 males, 22–59 years of age) (Fig. 2). The LAI was not modified by the drug in the absence of tissue extract.

The phagocytosis of yeast cells by monocytes was also significantly enhanced by lynestrenol at the two highest concentrations (10^{-1} and 10^{-2} μg/ml) studied in 6 normal subjects (3 females and 3 males, 22–59 years of age) (Fig. 3).

In all tests (rosette, LAI and phagocytic assays) differences in age and sex had no effect on the results obtained.

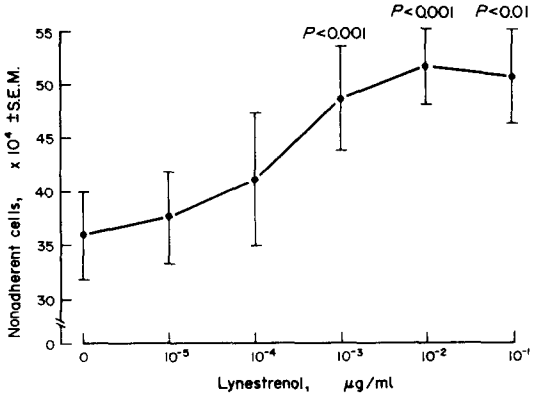

Fig. 2. Effect of various concentrations of lynestrenol on the number of nonadherent cells in the leucocyte adherence inhibition test (mean of 8 experiments ±S.E.M.)

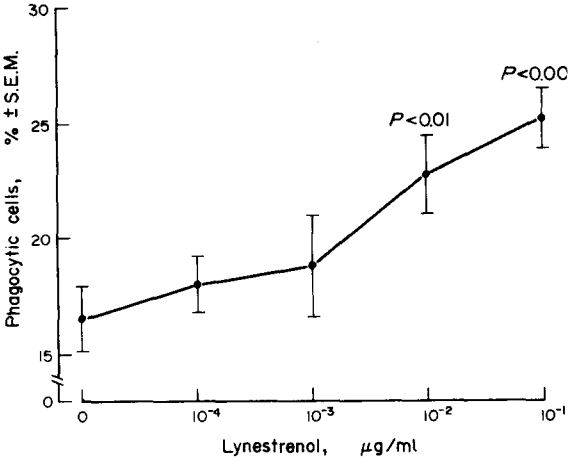

Fig. 3. Effect of various concentrations of lynestrenol on yeast phagocytizing monocytes (mean of 6 experiments ±S.E.M.)

DISCUSSION

The present study was undertaken to evaluate the influence of lynestrenol upon various, *in vitro*, immunological tests using human blood cells from healthy volunteers. In a previous report, it was shown that this agent enhances (up to 100%) the proliferative response of human lymphocytes in the presence of phytohemagglutinin at lynestrenol concentrations between 10^{-3} and 10^{-1} μg/ml as well as in the mixed leucocyte culture reaction at concentrations of 10^{-2} and 10^{-1} μg/ml. In contrast, lynestrenol did not show any effect on pokeweed mitogen stimulation. Moreover, lynestrenol by itself had no stimulatory affect on lymphocytes (Wybran *et al.*, 1977). On the basis of these experiments, it was thought that lynestrenol is able to modulate and amplify mainly T-cell responses. In view of this interpretation, it was of interest to determine if lynestrenol could also affect other immune functions.

Lynestrenol significantly increased the percentage of active T cells without affecting the percentage of total T rosettes (Fig. 1). This increase was detected at concentrations between 10^{-4} and 10^{-1} μg/ml being most marked at 10^{-1} μg/ml. This concentration range is therefore very similar to that observed to be effective in the lymphocyte proliferation system. These results provide further direct evidence that lynestrenol affects normal T-cell function. Indeed, active T rosettes are thought to represent a T-cell subpopulation actively involved in cell-mediated functions (Wybran *et al.*, 1973). The correlation between the percentage of TEa and the level of cell mediated immunity is based on both *in vitro* and *in vivo* data. Patients with Wiskott-Aldrich syndrome may show clinical and immunological improvement after transfer factor treatment (Wybran *et al.*, 1973) in association with increases in the percentage of active T rosettes without TEt modification. Similarly, melanoma patients treated with BCG may show increased cell mediated functions as well as an augmentation in the percentage of blood TEa (Wybran, Spitler, Lieberman & Fudenberg, 1976). Finally, it has been shown that the status of cell mediated immunity correlates directly with blood

TEa in patients with various types of immunodeficiency diseases (Horowitz, Groshong, Albrecht & Hong, 1975). Direct *in vivo* evidence has been provided by the observations of Felsburg *et al.* (1976). Normal subjects challenged intradermally with an antigen to which they are sensitized show an increase in their blood TEa without TEt modification. In contrast, the blood TEa did not vary in nonsensitive patients (Felsburg *et al.*, 1976). These results clearly indicate that the reaction to an antigen is associated with the active T-cell population. The *in vitro* data corroborate these observations. When antigen is added in culture to human leucocytes, one observes very rapidly an increase in TEa among the cultured lymphocytes if these leucocytes are sensitized to the antigen as judged by lymphocyte stimulation (Felsburg & Edelman, 1977).

The action of lynestrenol on the T-cell populations appears to be very similar to transfer factor, thymosin and isoprinosine (Holzman & Lawrence, 1977; Wybran, Levin, Fudenberg & Goldstein, 1975; Wybran, Govaerts & Appelboom, 1978). All these agents in common have immunostimulatory properties and the capacity to increase TEa without affecting TEt. Such comparative observations suggest that lynestrenol is also an immunomodulating agent.

The increase in red cell T rosettes with lynestrenol also indicates a direct action on a T-cell subset (Lambermont *et al.*, 1977). The functional rôle, if any, of this T-cell subpopulation is not yet clearly defined, so the action of lynestrenol in this rosette system is difficult to interpret. It is interesting to notice, however, that these autologous red cell rosettes are decreased in cancer patients (unpublished results).

The absence of effect of lynestrenol on EAC rosettes suggests that lynestrenol does not affect B-cell function. This observation is consistent with the absence of an enhancing effect of lynestrenol on pokeweed mitogen stimulation.

In other experiments, the influence of lynestrenol on the leucocyte adherence inhibition test was investigated. In this assay, one observes loss of adherence to glass tubes of both monocytes and neutrophils in the presence of a tissue extract to which the donor is sensitized (unpublished observations), compared to a tissue extract to which the donor is not sensitized (Grosser *et al.*, 1975; Appelboom *et al.*, 1978). The sensitization can be studied directly using the leucocytes of a sensitized donor (e.g. leucocytes of a rheumatoid patient in the presence of synovial extract) or indirectly using normal leucocytes preincubated with a serum containing a cytophylic IgG directed against the extract tested (e.g. serum of a cancer patient and tumor extract). The characterization of the cell type involved in the test is not clear. Our current work indicates that sensitized mononuclear cells activated by an antigen will release a humoral factor which decreases the adherence of polymorph neutrophils to glass tubes (unpublished observations). The present work indicates that lynestrenol increases the number of cells nonadherent to glass tubes in the presence of a tissue extract to which normal leucocytes are presumably not sensitized (Fig. 2). However, since the tissue extract is not characterized, it is possible that it contained contaminating antigens to which normal leucocytes may be sensitized. Therefore lynestrenol either induced in nonsensitized leucocytes a behaviour similar to the sensitized state or increased their sensitivity to contaminating antigens. However, it remains possible that the drug acts by a non-specific mechanism such as a direct membrane effect. Since isoprinosine, another immunostimulant, also increases the number of nonadherent cells in the LAI, one favors the specific hypothesis (Wybran *et al.*, 1978). The present experiments do not clearly indicate the target cells of lynestrenol action in the LAI. In conclusion, lynestrenol appears to be an agent capable of modifying leucocyte function in the dose range of 10^{-3} to 10^{-1} μg/ml.

This hypothesis is corroborated by the last set of experiments showing that lynestrenol increases the phagocytic properties of monocytes. Similar results have been obtained with isoprinosine (Wybran *et al.*, 1978). Thus, lynestrenol enhances an important defense mechanism like phagocytosis in a dose range similar to that effective in the leucocyte adherence inhibition assay.

A possible cellular interpretation for the effect of lynestrenol is that this drug increases the intracellular level of cyclic GMP. Indeed elevated cyclic GMP have been associated with increases in lymphocyte proliferative response, in active T rosettes (without TEt modification) and in leucocyte functions like chemotaxis (Hadden, Hadden, Johnson & Johnson, 1975; Galand, Lundak & Eaton, 1976; Sandler, Gallin & Vaughan, 1975).

Based on the *in vitro* data, we studied the effect of lynestrenol in hamsters inoculated with cells transformed by Herpes simplex virus type 1 (Wybran & Thiry, 1978). We showed that daily intraperitoneal injection of lynestrenol significantly delayed the appearance of tumor growth in treated animals and slightly increased their survival. This effect appears to be related to immunological mechanisms for several reasons: (1) levamisole, another immunomodulating agent also slightly delays the appearance of tumor growth, (2) lynestrenol had a synergistic protective effect with *Staphylococcus aureus* Cowan

A, a possible B-cell stimulatory agent, (3) lynestrenol inhibited the tumor facilitation effect produced by prior inoculation of dead cells; and, finally, (4) the lynestrenol-protected animals had an increased resistance to tumor challenge.

In conclusion, lynestrenol has shown an *in vitro* enhancing effect upon normal human T-lymphocytes, monocytes, and probably neutrophils. These results suggest that lynestrenol, provided it is used at the right concentrations, has immunostimulatory properties. These conclusions are supported by our earlier *in vivo* data (Wybran & Thiry, 1978) showing that lynestrenol delays malignant tumor growth.

Acknowledgement—This work was financially supported by the Fonds de la Recherche Scientifique et Médicale. We thank Dr Eric Van Bogaert for his helpful discussions.

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