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IMMUNOMODULATORY EFFECT OF THE HOMOEOPATHIC DRUG ENGYSTOL-N ON SOME ACTIVITIES OF ISOLATED HUMAN LEUKOCYTES AND IN WHOLE BLOOD

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ABSTRACT

Engystol-N at the doses of 10^{-4} and 10^{-8} in isolated human leukocytes stimulates the superoxide anion generation by neutrophils and the cytokine(s) production by T lymphocytes. In whole blood the same concentrations of the drug produce the decrease of the superoxide anion generation of neutrophils, this inhibiting activity appears 6 h after the administration of the drug and persists only in presence of lymphocytes. Culture media of T lymphocytes treated with Engystol-N show the same inhibiting effect on superoxide anion generation by neutrophils. From these data it is possible to conclude that the drug stimulates the secretion of lymphokine(s) with inhibiting action on superoxide anion generation of neutrophils that prevail over the direct stimulating effect, confirming and extending the immunomodulatory ability of the drug.

INTRODUCTION

Engystol-N is a complex homoeopathic drug generally used as an immunomodulator agent in many european countries; it is composed of vegetable extract from Vincetoxicum hirundinaria plant and sulfur. In vials

containing 1.1 ml of fluid, the active substance is the Vincetoxicum extract present in three equally distributed concentrations (10^{-6}, 10^{-10} and 10^{-30}) of 6.6 µl each obtained from a mother solution after 1:10 proportional dilutions with 30% ethanol solution and "dynamized" with ten vertical shakes, the sulfur is present as potentiating agent in two concentrations (10^{-4} and 10^{-10}) of 3.3 µl each and likewise dynamized. Current investigations have shown that Engystol-N exerts the following effects on immunocompetent cells: increases phagocytosis; in vitro clearance of the carbon; chemoluminescence in the phagocytizing cells Engystol-N has no effect on T cells transformation, IgG production, haemoglobin concentration, red and white cells number in the blood, phosphatase and transaminase enzymes (1, 2). In vitro and in vivo increases the migration of granulocytes and decreases their superoxide anion generation in asthmatic patients (3). These studies clearly showed the immunomodulatory activity of Engystol-N, but the effect of the drug on the relationship among the cell populations constituting the immune system are still unknown. The aim of this study was to help elucidate this relationship.

MATERIALS AND METHODS

Reagents: Hank's balanced salt solution, superoxide dismutase (SOD), cytochrome c, phorbol-12-myristate-13-acetate (PMA), zymosan, dextran, glutamine, Histopaque-1077, RPMI 1640, ethylenediaminetetraacetic acid (EDTA), fetal calf serum (FCS), bovine serum albumin, streptomycin, penicillin, fungizone and heparin were obtained from Sigma-Aldrich srl (Milan, Italy). Engystol-N was purchased from Guna srl (Milan, Italy). Other chemicals were of analytical HPLC grade.

Blood: human venous blood from healthy adult volunteers was collected with disposable plastic syringes and anticoagulated with 10 units of heparin per ml.

Isolation of neutrophils: peripheral blood was diluted 1:2 in Hank's solution, layered on Hystopaque-1077 and centrifuged at 400xg for 45 minutes at room temperature; the bottom washed twice in PBS was resuspended in 0.6% dextran then left for 45 minutes to allow erythrocytes to sediment, the clear erythrocytes shore was recovered, centrifuged at 1.800 rpm for 15 minutes and residual red cells were removed by hypotonic lysis as described by Haslett et al. (4). Cell viability determined by tripan blue exclusion was > 95%.

Isolation of T lymphocytes: after centrifugation over Histopaque as above, the opaque interface containing mononuclear cells was collected, washed twice in RPMI 1640, and monocytes depleted by adherence to plastic culture flasks for 1 h in 37°C 5% CO_2 humidified incubator. Supernatant containing the lymphocytes was centrifuged at 1.800 rpm for 15 minutes and the pellet resuspended in PBS supplemented with 0.5% of bovine serum albumin and 2 mM EDTA, T cells were magnetically separated from B cells by means of colloidal paramagnetic micro-beads conjugated to monoclonal mouse antibody anti CD19 surface antigen expressed on B lineage cells using positive selection columns ((Miltenyi Biotec, Bergisch Gladbach, Germany).

Lymphocytes cultures: T cells purified as above were placed in 24 flat-bottom well plates (Flow Laboratories, Irvine, Scotland) with RPMI medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml streptomycin, 2.5 μg/ml fungizone and were placed in a humidified incubator at 37°C and 5% CO_2; cell density was 5 x 10^5/well and viability determined by trypan blue dye exclusion was > 90%.

Mesaurement of superoxide anion release: O_2^- release was estimated using the superoxide dismutase-inhibitable reduction of cytochrome c assay described by

Bellavite et al. (5). Briefly: in plastic test-tubes were added 0.4 ml of Krebs-Ringer-Phosphate buffer (KRP) and 1.5 mg of cytochrome c, in the half of the tubes 50 μg of SOD and in one third 0.5 mg of zymosan or 0.5 μg of PMA; then 0.1 ml of blood or 10^6 neutrophils treated and untreated with Engystol-N. The final volume of the reaction mixture was 0.5 ml and reaction was allowed to proceed for 30 minutes at 37°C under continuous shaking. The incubation was stopped by the addition of 2 ml of ice-cold KRP and the tubes were centrifuged at 1.800 rpm for 5 minutes, the absorbance of the cell-free supernatants measured at 550 nm was determined by dividing the average difference in absorbance in samples tested with and without SOD by extinction coefficient for the reduced cytochrome c (0.0245) and multiplied for 2.5 (dilution factor). The results were expressed as nmoles of O_2^- / 10^6 granulocytes, on the basis of the total and differential counts of white blood cells.

Experimental protocols: Whole blood was subdivided into aliquot parts of 2 ml and preincubated with 10^{-4} or 10^{-8} final concentration of Engystol-N for times varying from 1 to 6 hours at 37°C under continuous shaking.

Isolated neutrophils were suspended in KRP at a concentration of 10^6 cells/ml then preicubated as whole blood for 6 hours.

Controls were incubated without drug in the same conditions.

T cells after 1 day of culture were treated with 1 μl of Engystol-N, culture media recovered 1, 2, 3, 4, 6 and 7 days after, centrifuged at 1.800 rpm to remove the cells and stored at –20°C.

Blood samples were centrifuged at 1.800 rpm for 10 minutes, the plasma separated from the cells, kept at room temperature and replaced with equal quantity of T cells culture media, then incubated for 1 hour at 37°C under continuous shaking, at last replaced with original plasma and submitted to

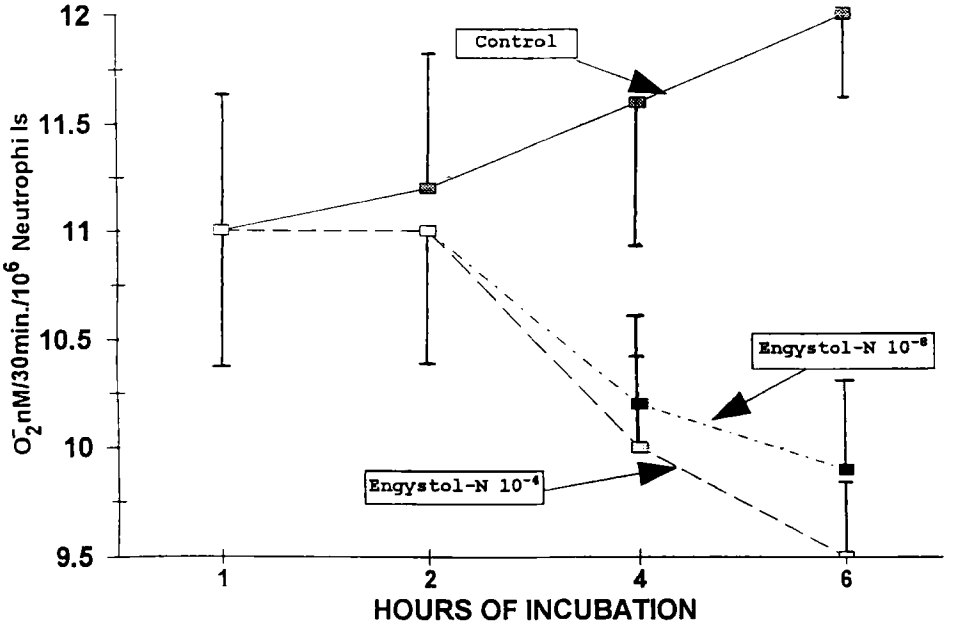

FIG. 1. Effect of 10^{-4} and 10^{-8} Engystol-N concentrations on superoxide anion generation by neutrophils in human whole blood after 1 and 6 hours of incubation using zymosan as stimulator.

superoxide anion release mesaurement. Controls were culture media of untreated T cells.

Statistical analysis: the data were analyzed by the unpaired Student's t test, significance was considered attained when $p \geq 0.05$.

RESULTS

The effect of Engystol-N at concentration of 10^{-4} and 10^{-8} on superoxide anion generation of the neutrophils in whole blood samples is shown in Fig. 1. After 1 and 2 hours of incubation any effect of the drug is not observable. Extending incubation, an inhibiting effect of about 13% appeared after 4 hours and achieved

21% after 6 hours. Two concentrations of the drug did not show significant differences in their inhibiting activity. This result was observed after zymosan stimulation.

Fig. 2 shows the effect of Engystol-N on zymosan- or PMA-induced O_2^- release by isolated human neutrophils. The cells were preincubated for 1 hour with two different concentrations (10^{-4} and 10^{-8}) of the drug. Anyhow Engystol-N showed a very high increase of O_2^- production of about 100%. In the histograms, the O_2^- release of control cells was set as 100%.

Fig. 3 shows the effect of Engystol-N on zymosan- or PMA-induced O_2^- release by human neutrophils in the presence of lymphocytes. The plasma and the largest number of red cells were removed from whole blood by dextran sedimentation. Clear share was centrifuged at 1,800 rpm for 15 min, pellet containing leukocytes (granulocytes + lymphocytes) washed twice in KRP buffer and residual erythrocytes removed by hypotonic lysis. The leukocytes were resuspended in KRP at a concentration of 10^6 cells/ml and preincubated with 10^{-4} and 10^{-8} final concentration of Engystol-N for 6 hours to conform it to whole blood treatment. Both concentrations of the drug produced a decrease of O_2^- release higher than 50% either from zymosan- or PMA-stimulated cells.

The effect of media from T lymphocyte cultures treated with 1 µl of Engystol-N for a week, on zymosan- or PMA-induced O_2^- generation is shown in Fig. 4. The culture media inhibited the O_2^- production zymosan-induced with maximal effect after 3 days of culture (62%) and return to the normality after 7 days. The inhibition on PMA-induced production initiated after 2 days of culture and reached the highest value after 4 days (78.3%) returning to the normality in 7^{th} day.

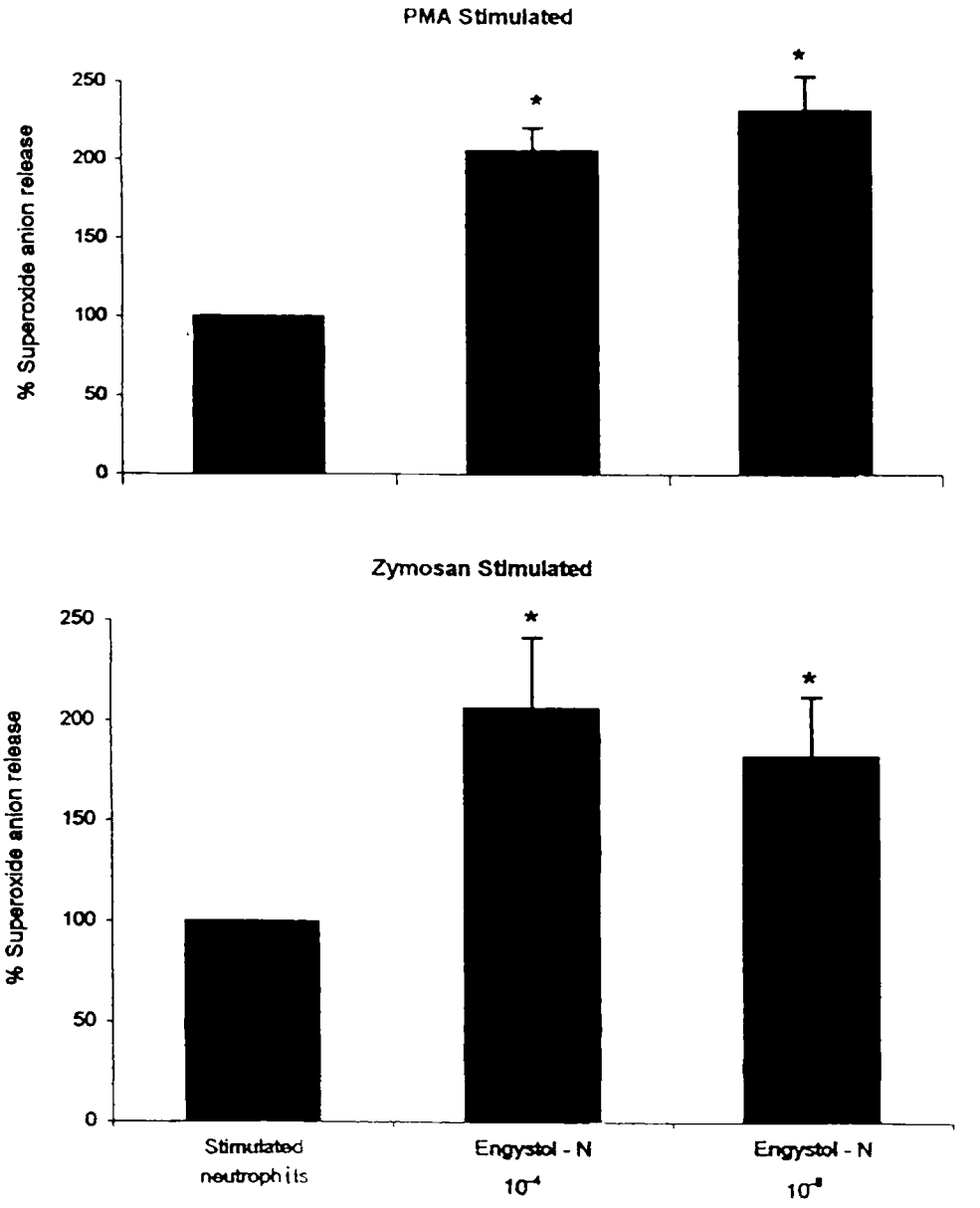

FIG. 2. Effect of Engystol-N on PMA- and Zymosan-induced O_2^- production by human neutrophils.

The cells were preincubated with 10^{-4} or 10^{-8} Engystol-N for 1 hour and then stimulated with PMA or zymosan for 30 minutes.

*Significantly different from control cells.

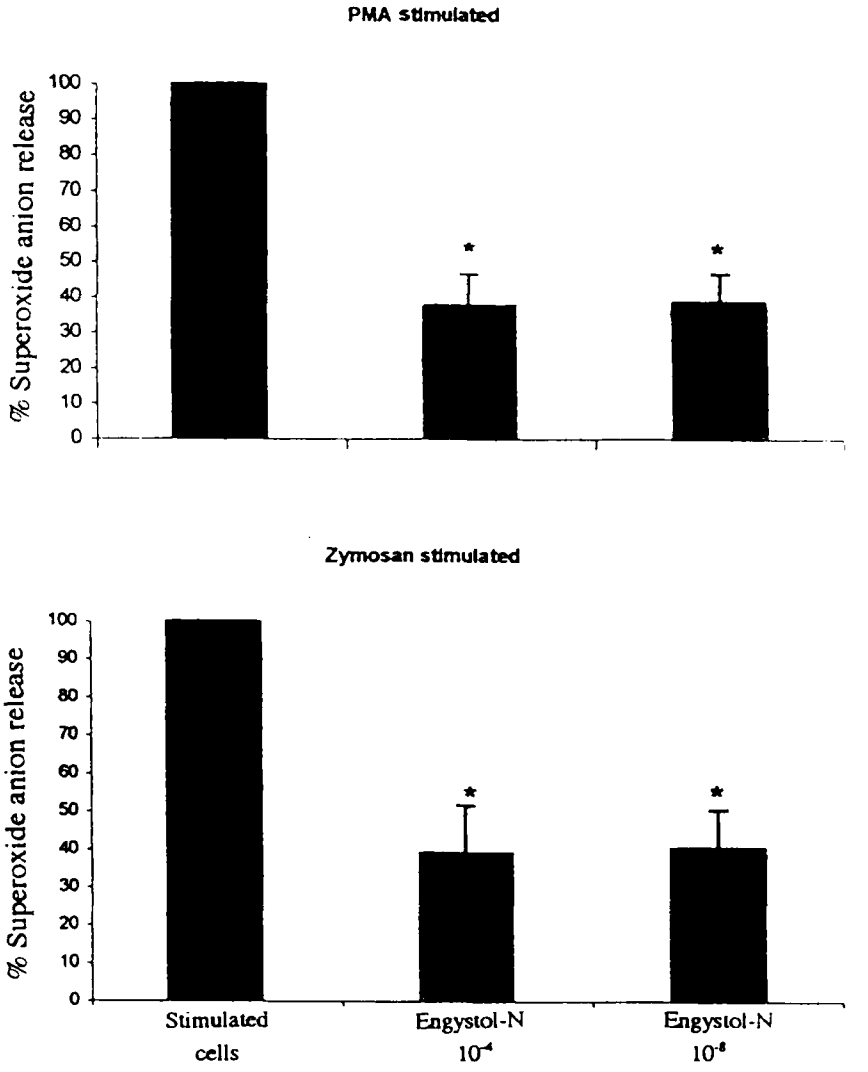

FIG. 3. Effect of Engystol-N on PMA- and zymosan-induced O_2^- production by human blood leukocytes (granulocytes + mononuclear cells).

The cells were preincubated with 10^{-4} or 10^{-8} Engystol-N for 6 hours and then stimulated with PMA or zymosan for 30 minutes.

*Significantly different from control cells. Results are expressed as mean values ± S.D. and were obtained from four independent experiments.

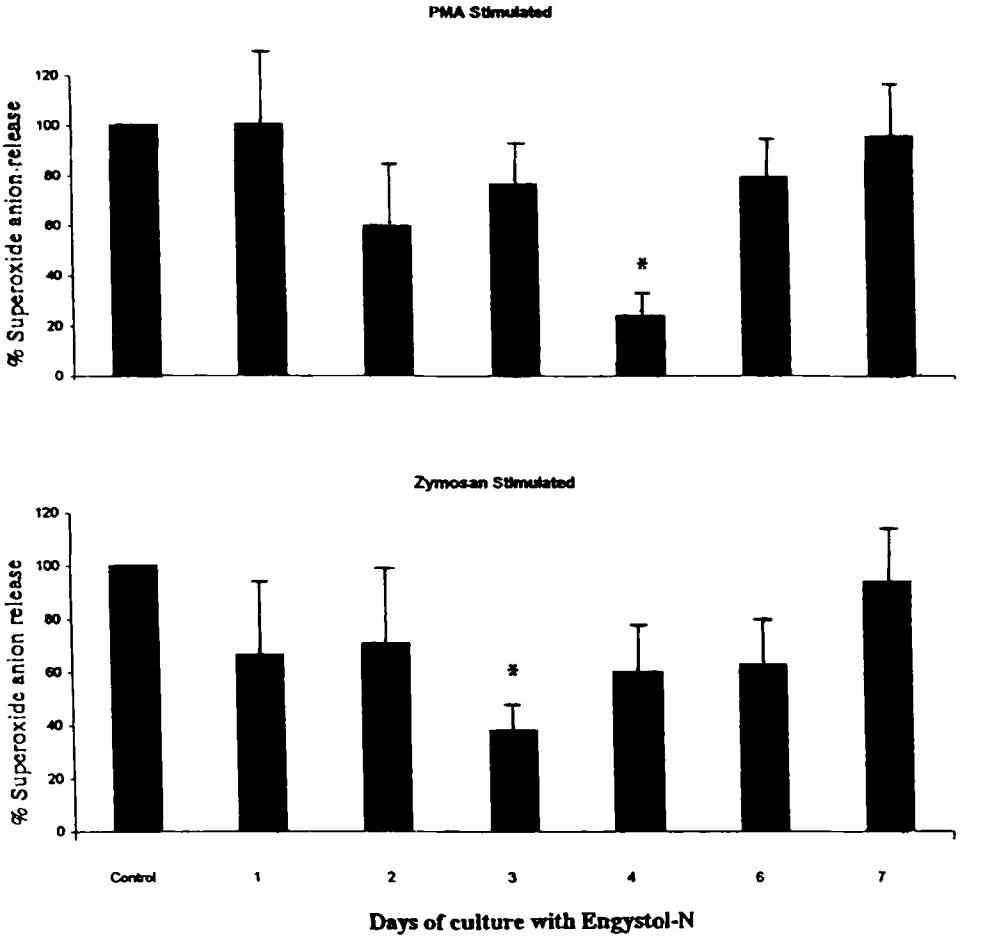

FIG. 4. Effect of culture media from T lymphocytes culture on PMA- or zymosan-induced O_2^- production by neutrophils in human whole blood.
Blood samples separated from plasma were preincubated with culture media for 1 hour, washed, replaced with original plasma and then stimulated with PMA or zymosan for 30 minutes.
*Significantly different from control cells. Results are expressed as mean values ± S.D. and were obtained from three independent experiments.

DISCUSSION

Engystol-N is a drug administrable by intravenous, intramuscular or oral route. Changing the supply route, the quantity of the drug absorbed can change. We estimated this variation included between the dilutions of 10^{-4} and 10^{-8}, and used these ones in our experiments (Fig. 1, 2 and 3). We did not observe significant differences in the behaviour of two different dosages concordantly with the principles of the homoeopathic-homotossicologic therapy, based on very high dilutions, "dynamization" with vigorous vertical shakes, and an effectiveness not dose-dependent (6-9).

To stimulate the superoxide anion generation by neutrophils we used a particulate (zymosan) or a chemical (PMA) stimulant, inasmuch as the superoxide production is the final event of the sequence of several steps involving plasma membrane (binding of the stimulant to receptor, formation of cytochrome b558, activity of FAD-containing flavoprotein) and cytosol (signal transduction, proteinkinase c activation, phosphorilation of phagocyte oxidase); using particulate stimuli all the steps are activated, using chemical stimuli the first steps are bypassed and proteinkinase c is directly activated. The results by us obtained (Fig. 1, 2 and 3) did not show significant differences of the superoxide anion production by neutrophils in different experimental conditions using particulate or chemical stimulant. This behaviour independent from the nature of the stimulus suggest the involvement of the terminal steps of superoxide production in the cytosol, specifically these following proteinkinase c activation and leading to cytochrome b558 formation.

Samples of whole blood after a brief incubation of 1 hour with Engystol-N, as shown in Fig. 1, did not displaye differences from control, but after 6 hours of incubation the inhibition of superoxide anion generation appeared. Similar

inhibition was observed by Matusiewicz (3) in asthmatic patients after therapy with Engystol-N if compared with the placebo group. These data appeared apparently contrasting with those of Wagner et al. (2) that observed increase of human neutrophils chemoluminescence after intravenous 5-days treatment with homoeopathic drugs including Engystol-N; chemoluminescence demonstrable in phagocyte cells isolated is in connexion with O_2^- and H_2O_2 productions. To clear up this discrepancy we isolated human neutrophils and tested for superoxide anion generation after treatment with Engystol-N for 1 hour. As shown in Fig. 2 the effect of the drug was highly stimulatory in these conditions according to Wagner et al. (2), consequently we concluded that in whole blood there is factor(s) that stimulated by Engystol-N thwarts the O_2^- generation, moreover inhibit it. We considered the possibility of plasmatic factor(s), therefore we removed plasma and red cells by dextran sedimentation and observed the behaviour of the neutrophils in presence of lymphocytes. As showed in Fig. 3 after 6 hours of incubation it is remarkable an inhibition of superoxide anion production higher than 50%, we concluded that lymphocytes produced factor(s) with inhibiting action on O_2^- generation of neutrophils. Since Wagner et al. (1) did not observe an effect of Engystol-N on IgG production, we excluded B lymphocytes and studied T cells isolated in culture in persence of 1 μl of Engystol-N. T lymphocytes are producers of lymphokines and to show their eventual implication, we cultivated T cells for a week and every day the culture medium was recovered and tested on whole blood as to O_2^- production by neutrophils. Results are showed in Fig. 4 where it is observable a constant inhibiting activity from day 1 to 6 after Engystol-N induction with maximal effect (statistically high significant) in day 3 and 4 respectively after zymosan and PMA stimulation. Similar behaviour remarked Wagner et al. (2) measuring during 11 days the phagocytosis index of neutrophils

from healthy volunteers receiving i.v. injections of Engystol-N within a 5-day treatment, inasmuch as the maximum of phagocytosis activity was reached between the 3th and 5th day of injection.

The difference of one day in the beginning of the inhibiting effect and in the reaching of the maximal activity observable in Fig. 4, could be due to different mechanism of stimulation exerted by a particulate or a chemical stimulant involving respectively the plasma membrane or direct proteinkinase c activation and/or to different cytokines produced by T cells in not coincident time.

The data obtained demonstrate that Engystol-N enhanced superoxide anion production by human isolated neutrophils after zymosan or PMA stimulation (Fig. 2) and stimulated cultivated human T lymphocytes to secrete lymphokine(s) with an inhibiting effect on superoxide anion production by neutrophils (Fig. 4). These simultaneous effects on neutrophils and T lymphocytes show that the drug modulate reciprocal relationships between two populations of immunocompetent cells. Its effects are not dose-dependent (Fig. 1, 2 and 3), consequently the drug is effective independently by administration route.

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