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EFFECT OF CLOVER EXTRACT ON PROLIFERATION OF HUMAN AND MURINE LYMPHOCYTES IN VITRO

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Synthetic and semisynthetic flavonoids dibenz-γ-pyrone derivatives are currently regarded as promising antitumor preparations whose action is associated both with a direct cytotoxic effect of tumor cells [5, 6] and with modification of the biological response, namely with stimulation of activity of natural killer lymphocytes in the absence and in the presence of interleukin-2 [4, 6, 7, 10]. Various species of clover are sources of natural flavonoids, and they accumulate the highest concentration of these biologically active substances in the flowering phase [2].

The aim of this investigation was to study the effect of an extract of biologically active substances isolated from red clover (*Trifolium pratense* L.) on the proliferative activity of normal human peripheral blood lymphocytes and mouse spleen cells.

EXPERIMENTAL METHOD

Extracts of biologically active substances of *T. pratense,* including total flavonoids, were obtained by treating the raw material with 80% ethanol, followed by heating on a waterbath for 5 min [1]. The alcoholic solution was subjected to freeze-drying. The extracts were obtained from plants in the flowering period (extract 1) and in the budding phase (extract 2). Healthy human peripheral blood mononuclears (MON) were isolated by the method in [3]. For this purpose, after centrifugation for 30 min at 450g and at room temperature, the cells which were concentrated above the Ficoll-Verografin layer with density of 1.077 g/cm^3 were harvested, washed twice, and suspended in medium RPMI 1640, enriched with 5% fetal calf serum (FCS). To obtain splenocytes of BALB/c mice (aged 3-4 months, weight 20-25 g, bred at the "Stolbovaya" Nursery, Moscow Region), the animals were killed by cervical dislocation, the spleens were removed, and a suspension of single cells was prepared in a Potter homogenizer in medium RPMI 1640 with 5% FCS. The human or murine cells ($5 \cdot 10^4$-$1 \cdot 10^5$) were cultured in 0.2 ml medium RPMI 1640 with 5% heat (56°C)-inactivated FCS, $2 \cdot 10^{-3}$ M L-glutamine, 5 mM HEPES ("Flow Laboratories"), $3 \cdot 10^{-5}$ M 2-mercaptoethanol ("Merck"), and 40 μg/ml of gentamicin in 96-well round-bottomed plates for 3, 4, and 5 days at 37°C and in a humid atmosphere containing 5% CO_2. The concentrations of clover extract were 0.04-2.5%. DNA synthesis was determined by measuring incorporation of ^{3}H-thymidine, added in a dose of 0.5 μCi to each

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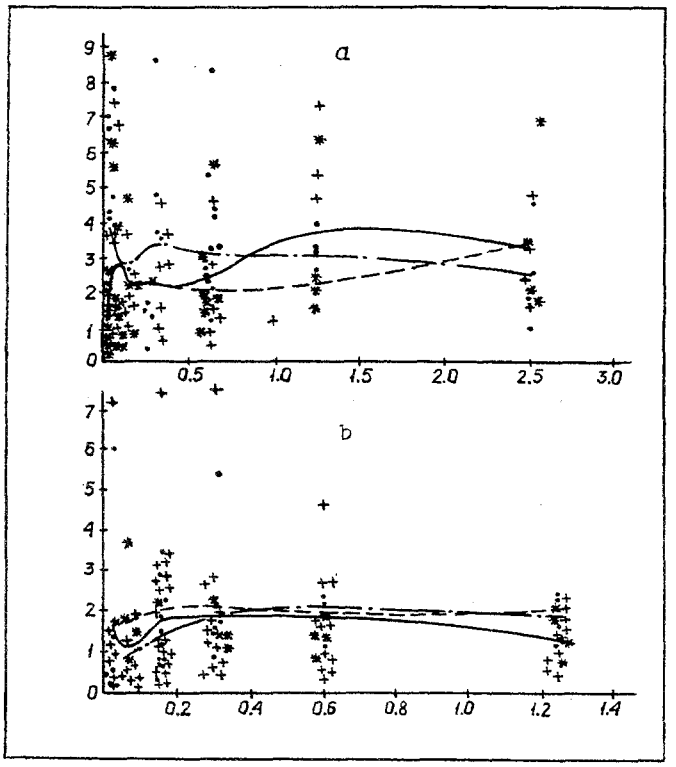

Fig. 1. Effect of extracts 1 (a) and 2 (b) of *T. pratense* on proliferative activity of healthy human peripheral blood lymphocytes. Thin line indicates 3rd day, bold line 4th day, broken line 5th day of incubation. Abscissa, dilution of preparation (in %); ordinate, index of stimulation (ratio of incorporation of label in experiment to control).

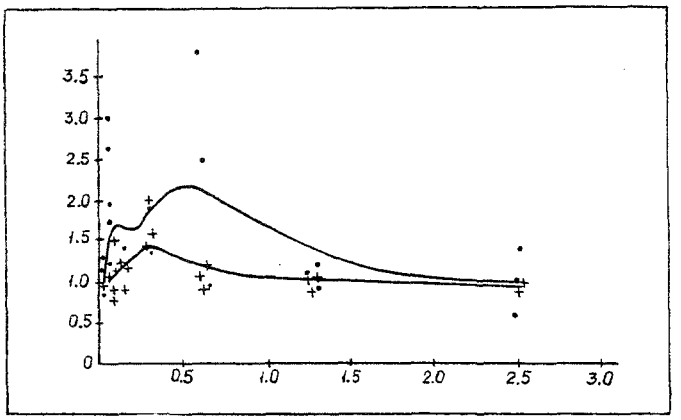

Fig. 2. Effect of extract 2 of *T. pratense* on proliferative activity of mouse splenocytes. Legend as to Fig. 1.

well of a 96-well plate 6 h before the end of the test. The duration of the proliferative test was 72, 96, or 120 h. The index of stimulation of proliferation was determined as the ratio of incorporation of label in the experiment to the control.

EXPERIMENTAL RESULTS

Extract 1, obtained from clover in the flowering period, in concentrations of 0.04 to 2.5% caused significant stimulation of DNA synthesis in human MON. A peak of proliferation was observed most often in a concentration of 0.5%, during the 3rd day of incubation: in concentrations over 1% – after the 4th day, in a concentration of 2.5% after the 5th day of incubation (Fig. 1a). Peaks of DNA synthesis in different donors also were observed after 3, 4, and 5 days of incubation, possibly connected with the initial level of the immune status of the test donors. Extract 2 had a weaker stimulating action of human MON; a maximum was observed most frequently in a concentration of 0.2% and it did not depend significantly on the duration of incubation (Fig. 1b).

The action of extract 2 on proliferation of mouse lymphocytes was less marked than that of human cells: after incubation for 3 days the index of stimulation reached a maximum of 2-2.5, but after 4 days significant stimulation was not found (Fig. 2).

It was shown previously that maximal accumulation of flavonoids in clover is observed during the flowering period [1, 2], and this definitely correlated with the results, for activity of extract 1, obtained from clover in the flowering phase, was higher than that of extract 2, obtained in the budding phase.

Extract of *T. pratense,* the active principle of which is total natural flavonoids, thus stimulates proliferation of human and murine lymphocytes. The action of the preparation may be associated with the known ability of flavonoids to inhibit membrane ATPase, to inhibit the cyclooxygenase pathway of arachidonic acid metabolism, and to affect gene expression and levels of intracellular Ca^{2+} and cAMP [8-10].

The stimulating effect of extract of biologically active substances of *T. pratense* on lymphocyte proliferation demonstrates the need for further research into the value of this preparation as an antitumor and anti-inflammatory agent.

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