# Factors Influencing the Immunomodulating Effect of Met-Enkephalin: Role of Mitogen Dose, Stage of Cell Activation, and Time of Opioid Administration

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Met-enkephalin in a concentration range of $10^{-15}$ to $10^{-9}$ M exhibited an immunomodulating effect upon concanavalin A-induced proliferation of mouse lymphocytes *in vitro*. The effect of met-enkephalin was shown to depend on the stage of lymphocyte activation, the time of opioid administration, and the dose of mitogen. Met-enkephalin produced the maximal effect when given in the phases of increase or decrease of the proliferative response. Met-enkephalin augmented the proliferative response to the suboptimal dose of concanavalin A and, on the contrary, inhibited the response to the optimal dose of mitogen. Administration of met-enkephalin at different times could both inhibit and stimulate proliferation depending on the stage of lymphocyte activation. An inhibitory effect was induced by δ-class opioid ligand, while κ-ligand was responsible for stimulation. Naloxone abolished the stimulating effect of opioids.

**Key Words:** *opioid peptides; lymphocyte proliferation; stage of cell activation*

Opioid peptides belonging to the class of neuro- and immunomodulators exert numerous effects on the immune system [3,10-13] and are therefore of considerable scientific interest. The action of opioids is mediated via specific receptors of the μ-, δ-, and κ-types, the presence of which on cells of the immune system has been established in a number of studies [2,4-6].

Analysis of the immunomodulating effect of opioids on a model of cell proliferation is frequently complicated by contradictions of the data obtained. For example, it is known that endorphins and enkephalins can both inhibit and stimulate the proliferative response of lymphocytes in the same range of concentrations [10,12-14]. Such opposite effects are primarily due to differences in the test systems used. One of the factors governing the pattern of opioid effect on the proliferative response of lymphocytes *in vitro* is the dose of mitogen. For example, the immunostimulatory effect of dalargineis more pronounced against the background of a suboptimal concanavalin A (ConA) concentration [1]. Leu-enkephalin stimulates proliferation induced by a low dose of phytohemagglutinin (PHA) but inhibits high dose PHA-induced proliferation [13].

The time of opioid administration also plays an important role in the nature of the effect. Dynorphin heightens the T-cell response to PHA when administered 48 hours later, but not before or simultaneously with the mitogen [3]. The effect of opioids on cell immunity is characterized by an intricate concentration dependence. Thus, met-enkephalin (ME) in a concentration of $10^{-13}$ M stimulates antigen-induced lymphocyte proliferation; however, in a concentration range of $10^{-11}$ to

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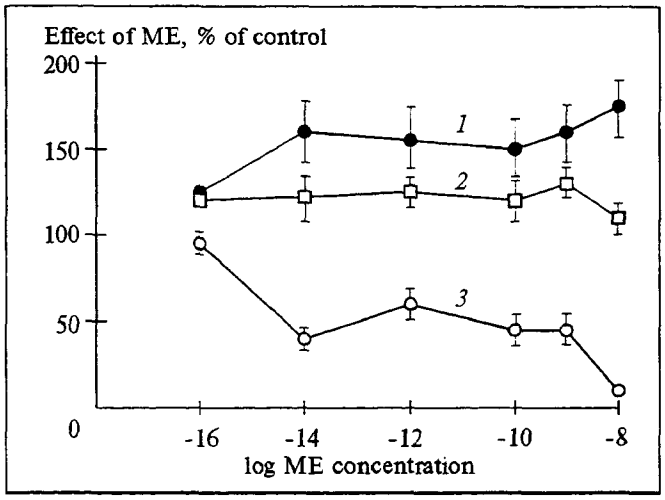


**Fig. 1.** Immunomodulatory effect of ME on proliferative response of mouse lymphocytes to ConA in different doses. ConA concentration: 2 μg/ml (*1*) and 10 μg/ml (*3*). *2*): ConA in a dose of 2 μg/ml + ME + naloxone ($10^{-6}$ M).

$10^{-7}$ M it inhibits the response to the antigen [11]. The initial state of the cells and the functional state of the organism as a whole also influence the immunomodulatory effect of opioids [9,14].

Thus, unraveling the mechanism of opioid-mediated immune response regulation represents a complex problem calling for the analysis of diverse data obtained in different experimental systems. The object of the present investigation was to study the role of a set of factors, such as mitogen and opioid concentration, time of opioid administration to the culture of mitogen-activated lymphocytes, and stage of cell activation, in the realization of the ME-mediated immunomodulatory effect.

## MATERIALS AND METHODS

The experiments were carried out on female $F_1$ (CBA×C57Bl/6) mice weighing 18-22 g. The following opioid ligands were used: ME; specific ligands of μ-type receptor ([d-Ala$^2$,N-Me-Phe$^4$-Gly$^5$-ol]-enkephalin - DAGO); of δ-type receptor ([dSer$^2$-Leu$^5$-Thr$^6$]-enkephalin - DSLET); and of κ-type receptor ([5a,7a,8b(-)-N-methyl-N-[7-pyrrolidinyl)-1-oxopyro(4,5)-8-yl]-benzoacetamide - U69.593). Naloxone (an antagonist of opioid receptors) in a concentration of $10^{-6}$ M was also used.

The effect of opioids on the lymphocyte proliferative response was studied *in vitro*. Popliteal and axillary lymph nodes were homogenized in RPMI 1640 medium. Cells were washed twice by centrifugation for 10 min at 600 g and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mM HEPES buffer, and 100 μg/ml gentamicin. The cell concentration was adjusted to $10^6$/ml. Proliferation was induced by the T-cell mitogen ConA added to the final concentration of 2 and 10 μg/ml followed by incubation at 37°C in an atmosphere with 5% $CO_2$. At various times after the start of incubation $^3$H-thymidine was added to the wells of microplates in a dose of 0.5 μCi per well, after which the cells were further incubated for 4 hours under the same conditions.

Opioids in the concentration range $10^{-16}$ to $10^{-8}$ M were introduced simultaneously with mitogen or 4 hours before assessment of the level of proliferative response. In certain cases the culture medium was replaced with fresh before opioid introduction.

The proliferative response was evaluated by recording $^3$H-thymidine incorporation. For this purpose cells were transferred to GF/C filters using a semiautomatic harvester. Radioactivity was measured in a Rack-beta counter. The count efficiency was about 32-34%. The effect of opioids was calculated as the ratio of ME-treated to control (mitogen only) cell culture radioactivity.

The experimental data were processed using Sigma Plot 5.01 graphic software. Statistical analysis was performed using Student's *t* test at $p<0.05$ for 3-6 replications at each experimental point.

## RESULTS

For evaluating the immunomodulatory effect of ME on lymphocyte proliferation, a dose of $10^{-16}$ to $10^{-8}$ M was added to the cell cultures simultaneously with ConA in doses of 2 μg/ml and 10

**Table 1.** Effect of Selective Opioid Agonists on the Proliferative Response of Mouse Lymphocytes (%)

| Ligand | 24 h after activation | | 96 h after activation | |
|---|---|---|---|---|
| | $10^{-9}$ M | $10^{-15}$ M | $10^{-9}$ M | $10^{-15}$ M |
| DAGO | 111 | 110 | 175 | 134* |
| DSLET | 73* | 73* | 136 | 166 |
| U69.593 | 104* | 110 | 261* | 264* |
| ME | 75* | 116* | 178* | 208* |

**Note.** *: The differences are significant ($p<0.05$).

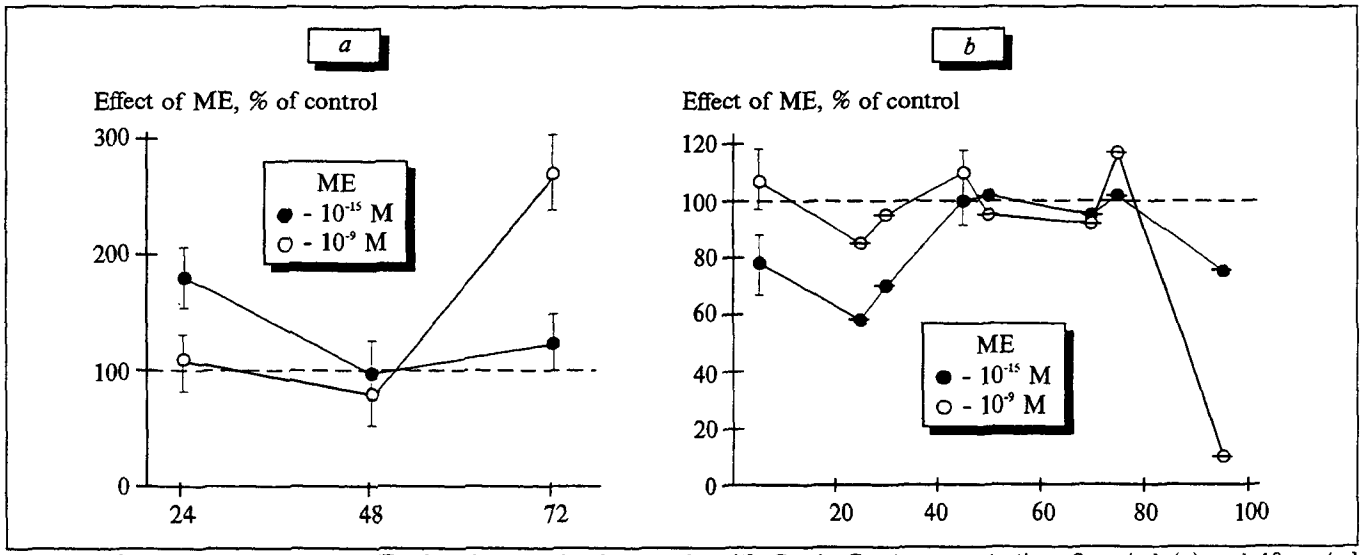


**Fig. 2.** Dynamics of effect of ME administered simultaneously with ConA. ConA concentration: 2 μg/ml (*a*) and 10 μg/ml (*b*). Abscissa: time of proliferative response, hours.

μg/ml. Preliminary titration had shown these mitogen doses to be suboptimal and optimal, respectively. The effect of the opioid was assessed 72 hours later. As is shown in Fig. 1, ME administered in a concentration range of $10^{-14}$-$10^{-8}$ M augmented the effect of the suboptimal dose of ConA. The effect of ME varied within 60-80% against the controls. On the other hand, in cultures with the optimal dose of mitogen the same concentrations of ME caused proliferation inhibition by 40-60%. On the whole, these data are in agreement with those in the literature [13] and provide experimental confirmation of the hypothesis concerning the role played by the degree of cell activation in the opioid-mediated immunomodulatory effect [1]. Naloxone, a classical opioid receptor antagonist, abolished only the stimulating effect of ME, indicating that different types of receptors are involved in the realization of opioid-induced stimulating and inhibitory effects on the proliferative response of lymph node cells.

In further experiments we used ME in 2 concentrations, $10^{-15}$ M and $10^{-9}$ M. The first (minimal) concentration served as a threshold one which was expected to yield reproducible results, and the second one was chosen as one of the standard receptor concentrations. In order to compare the immunomodulatory effect of ME at different stages of the cell response, we followed the kinetics of proliferation induced by the suboptimal and optimal ConA doses administered simultaneously with ME. With a ConA concentration of 2 μg/ml ME induced maximal stimulation 1 and 3 days after the start of culturing (day +1 and day +3) (Fig. 2, *a*), and a with ConA concentration of 10 μg/ml the maximal inhibitory effect of ME was observed 1 and 4 days after the start of culturing (Fig. 2, *b*). Against the background of the optimal ConA dose ME-induced suppression attained 40% and 17% for the use of ME in concentrations of $10^{-15}$ M and $10^{-9}$ M, respectively. On the 4th day of cell culture the opposite relationship between ME concentration and inhibitory effect was observed, i.e., $10^{-9}$ M ME produced a stronger effect (90% inhibition) than $10^{-15}$ M (25% inhibition) (Fig. 2, *b*). ME-mediated enhancement of suboptimal mitogen dose-induced proliferation revealed a similar relationship to the opioid concentration: $10^{-15}$ M ME led to 80% stimulation, and $10^{-9}$ M ME to 20% stimulation on day +1; on day +3 stimulation was equal to 30% and 170%, respectively (Fig. 2, *a*). Such a concentration dependence may imply the action of receptors with different affinity [2,4-6], that control different phases of proliferation. Here it is worth noting once more that ME was introduced simultaneously with ConA. Peptides are known to be degraded within minutes by proteases present in the *in vitro* culture [8]. Therefore, the observed effect of ME may be connected with effect of the opioid on the induction of the mitogenic signal. It follows from the data presented that effect of opioids manifests itself mainly during the days corresponding to the rise and fall of the cell response. It may be assumed that in this particular case the regulatory action of the opioid had to do with the cellular signal systems responsible for triggering and halting the activation program.

Apart from this, opioids can have an effect when introduced into a lymphocyte culture at different phases following cell response triggering by mitogen. For an analysis of this effect, ME was

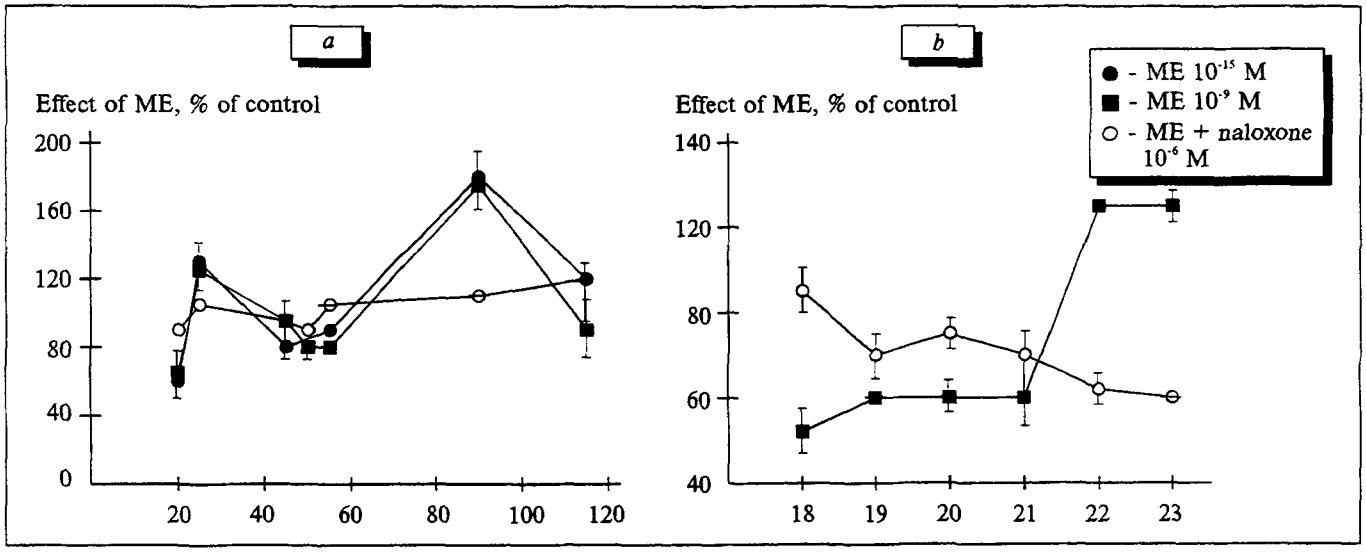


**Fig. 3.** Dependence of ME effect on time of administration to culture of activated lymphocytes. Abscissa: time of ME addition after induction of proliferative response, hours. *a*) study of ME effect in the period from day +1 to day +5; *b*) detailed study of ME effect inversion on 1st day of proliferation.

added at different times after proliferation induction by 10 μg/ml ConA and its effect was estimated 4 hours after addition (Fig. 3, *a*), at each time-point. It is to be noted that the maximal effect was observed mainly in the phases of rise and fall of proliferation, resembling the situation with simultaneous ConA and ME administration. Introduction of opioid peptide in the 20th hour of culturing inhibited $^3$H-thymidine incorporation by 35%; conversely, incorporation was stimulated by 30% when ME was added in the 24th hour. Administration of ME on day +4 caused a two-fold rise of the proliferative response as compared to the control. Both ME concentrations used produced a similar effect. The inversion of the ME effect observed during the 1st day was confirmed by a detailed kinetic study in which ME was added each hour during between the 18th and 23rd hour (Fig. 3, *b*). Thus, opioids can act both in the inductive phase of the immune response and at later stages of its development. Based on the data obtained in this part of the study, we may conclude that opioids may be involved in sending a secondary signal of the activation program. It is during this period that various regulatory factors, such as interleukins and interferons, actively accumulate and the corresponding receptors are synthesized, and opioids participate in these processes [7,12].

To determine the opioid receptor types mediating the observed effects of ME, we studied the effect of different opioid agonists on lymphocyte proliferation. Ligands selectively reacting with μ-, δ-, and κ-type receptors (DAGO, DSLET, and U69.593, respectively) and ME were added to cell cultures in concentrations $10^{-15}$ M and $10^{-9}$ M. The response was estimated 4 hours later. Preparations were added on days +1 and +4 (Table 1). In order to avoid a possible influence of endogenous opioids that are synthesized during culturing [15], preparations were introduced after washing of the cell monolayer and replacement of the culture medium with fresh. DSLET (δ-ligand) and ME administered on day +1 inhibited the mitogen-induced proliferative response by 25-27%. U69.593 (κ-ligand) administered on day +4 increased $^3$H-thymidine incorporation by more than 2.5 times as compared to the control. Possibly, processes connected with opioid stimulation of the lymphocyte proliferative response are mediated via κ-receptors, while δ-receptors transmit signals causing an inhibitory effect.

Thus, opioid compounds play an important role in the regulation of immunity, being involved in the triggering and halting of the cell-mediated immune response. In the present study we have shown that opiate-like substances can both control the mechanisms connected with proliferation induction and participate in the regulation of the secondary activation signal. The nature of the immunomodulatory effect depends on the phase of the proliferative response. The direction of the opioid effect is related to the degree of activation of the system. Moreover, the observed opposite effects imply the expression of different types of receptors depending on the magnitude of the response. The results provide evidence that opioids are recruited in paracrine and autocrine regulation of immune system functioning. This paves the way for their use as multipurpose immunocorrective agents.

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# Role of Various Murine B-Lymphocyte Subpopulations in the Immune Response to T-Independent Antigens

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Polyclonal antibodies to Lyb $5^+$ antigen of murine B lymphocytes are obtained and a methodological approach to the detection of cells carrying this antigen is developed with the aim of investigating the role of various subpopulations of mouse B lymphocytes in polyclonal activation induced by T-independent type 2 antigen. Hybridomas producing anti-Lyb 5.1 antibodies are obtained.

**Key Words:** *B lymphocytes; surface antigens; antibodies to Lyb 5 antigen*

As a rule, upon challenge an antigen not only induces antibody production in an animal organism, but intensifies the synthesis of nonspecific immunoglobulins (Ig) and increases the number of cells producing them. During a T-dependent response this latter circumstance depends largely on the nonspecific stimulating action of T lymphokines produced by antigen-activated T helpers. The mechanism of the production of antigen-induced nonspecific Ig in a T-independent response is unknown.

T-independent antigens are subdivided into type 1 antigens, polyclonal activators of B lymphocytes stimulating antibody production in both Lyb $5^-$ and Lyb $5^+$ B cells, and type 2 antigens, which are not mitogenic and are capable of inducing antibody production only in mature B cells carrying the Lyb $5^+$ marker [4]. That is why the appearance of not only antibody producers, but of numerous nonspecific Ig producers under the effect of some T-independent type 2 antigens has piqued the curiosity of scientists [3].

It is not clear whether only mature B lymphocytes with a surface Lyb $5^+$ marker are "triggered" to undergo proliferation and differentiation, or whether the B cells with the Lyb $5^-$ phenotype are also involved. Polyclonal activation induced by T-independent antigens shows some specific features distinguishing it from polyclonal activation caused by T-dependent antigens. It is possible that

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