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INFLUENCE OF LEVAMISOLE, NITROGRANULOGEN AND DIETYLDITHIOCARBAMATE ON THE ACTIVITY OF ECTO–ATPase FROM RATS LYMPHOCYTES

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Abstract: Extracellular membrane associated ecto–ATPase participates in the regulation of ATP and its degradation products levels in extracellular medium. These metabolites serve as information carriers and participate in cells interactions and therefore their concentration must be very precisely regulated. Application of immunomodulators in therapy requires studies on their influence on enzymes participating in nucleotides level regulation in immune system. The studies were conducted on rats immunized with SRBC, administered single or four doses of immunomodulators after immunization. The lymphocytes from spleen were collected 120 hours after the injection of SRBC. In the inflammation state the increase in ecto–ATPase was observed for subpopulation T and B. The immunomodulators are changing the activity of the enzyme, but the effect depends on the dose and the frequency of their application. The effects are also different for both subpopulations of lymphocytes. Diethyldithiocarbamate administered in single and multiple doses caused the inactivation of ecto–ATPase in the case of lymphocytes B and activation in the case of lymphocytes T. Levamisole in multiple doses inactivates ecto–ATPase in the case of both types of lymphocytes. Nitrogranulogen – in single and multiple doses – inhibited the activity of the enzyme and in cytotoxic dose (600 μg/kg) inactivated the enzyme below the control value. The discrepancies between the results of *in vitro* and *in vivo* studies were observed. Levamisole in *in vitro* studies did not influence the activity of ecto–ATPase and in *in vitro* studies displayed inhibiting properties.

Keywords: Lymphocytes, ecto–ATPase, diethyldithiocarbamate, Levamisole, nitrogranulogen.

A correctly functioning immune system protects the organism against various infections, allergic and neoplastic diseases. This activity depends on the organism ability to maintain steady state by creating its own autoregulative mechanisms which are under the influence of nervous, endocrine and immune systems.

Manipulation of immune mechanisms becomes an important issue, especially after introduction of both natural and synthetic pharmacological modulators of immune response to therapy. Immunological problems related to the utilization of popular immunomodulators such as Levamisole (LMS), Diethyldithiocarbamate = Imuthiol (DTC), Nitrogranulogen = Mechloroethamine (NTG) are the subject of many „in vivo'' and „in vitro'' studies (1–6). However, the direct effect of these substances on cell membrane is still unclear (7,8). Cytoplasmatic membrane is a subject of particularly intensive attack of various substances, which while reacting with proteins and lipids evoke structural and functional changes in the cell.

The changes in the activity of membrane enzymes (ecto–enzymes) caused by various substances introduced into organism, influence the metabolic processes in individual cells. In order to study the effect of immunomodulators on the membrane of cells partaking in immune system, we chose one of the cytoplasmic membrane enzymes, participating in extracellular metabolism: ATPase.

Recent studies have demonstrated that ATP beside being energy donor is also serving as an information carrier. A similar role is ascribed to ATP degradation products such as ADP, AMP and adenosine (9,10). Because these compounds are controlling various, sometimes contradictory, processes, therefore, cells and tissues must be equipped with a special mechanism regulating their concentration. The compounds described above are substrates of numerous membrane enzymes, to which belong ATPases. ATPases utilize the energy of pyrophosphate bonds in syntheses and transport of metabolites and ions. Therefore, the changes in ecto–ATPase activity are of crucial consequences, especially in cells of immune system.

The earlier studies have confirmed that NTG, LMS, and DTC intensify the humoral response of organism to various thymodependent antigens. The

effects of their action depended on the dose and frequency of their administration (11–13).

In our work conducted on the model of rats stimulated with sheep blood cells (SRBC) we compare the effect of LMS, NTG and DTC administered repeatedly and in a single dose, at different time intervals from the time of SRBC administering on the activity of ecto–ATPase of lymphocytes T and B isolated from spleen.

MATERIALS AND METHODS

Reagents

ATP (disodium salt from equine muscle), carragenine, ethylenediaminetetraacetate (EDTA), NaN_3, Hepes, pNFP, Trypan blue, DMSO (dimethylsulfoxide), sodium diethyldithiocarbamate, Levamisole hydrochloride, Tris and PBS were obtained from Sigma Chemical Co., FSBA (5'–p–fluorosulfinylbenzoyladenosine) from Bayer, UK, PPMI 1640 medium from Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland. Nitrogranulogen (mechlorethamine hydrochloride) from Polfa, Poland. Fetal Calf Serum – from Gibco. Mouse antibody anti–Thy 1.1 (labeled with fluoroscein isothiocyanate – FITC) and mouse antibody anti CD45RA (labeled with FITC) – from SEROTEC. All other reagents were purchased from Boeringher.

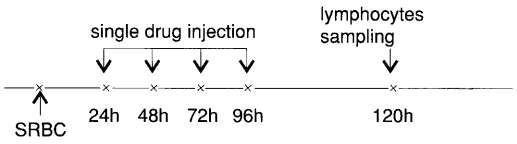
Experimental procedure

The studies were conducted on eight weeks old Buffalo rats. The rats were immunized by intraperitoneal administration of 0.5 ml of 10% suspension of erythrocytes (SRBC) that is 10^9 of erythrocytes per animal.

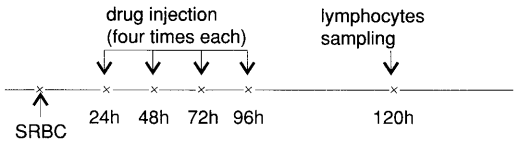
Levamisole (LMS) in a dose of 2.5 mg/kg, nitrogranulogen (NTG) in a dose 5 μg/kg or 600 μg/kg and diethyldithiocarbamate (DTC) in a dose of 20 mg/kg were injected intraperitoneally. Each dose was administered in a volume of 0.2 ml.
In every experimental design a control group (n=10) was included which constituted of rats receiving 0.2 ml of sterile buffered physiological solution per animal instead of the drugs.

Two methods of drug administration were employed. In the first – each LMS, NTG and DTC were administered in one dose at 24^{th}, 48^{th}, 72^{nd} or 96^{th} hour after immunization. The lymphocytes from spleen were collected 120 hours after immunization (Scheme 1).

In the second method all three drugs were administered four times at 24–hour intervals from the moment of immunization (Scheme 2).



Scheme 1.



Scheme 2.

Isolation of lymphocytes T and B

The spleens taken from rats were suspended in RPMI 1640 containing 2% fetal serum, placed on ice on Petri dishes and mechanically dispersed through a nylon mesh. The suspension was filtrated through a syringe filled with cotton wool, what allowed to eliminate clumps from separate cells. The suspension was centrifuged for 10 minutes at 400g (Heraeus Omnifuge 2.0 RS), cell pellet was resuspended in cold PBS containing 0.5% NaN_3 (the procedure was repeated three times). Subsequently, the pellet was suspended in PBS. Equal or multiple volumes of isotonic 0.2% trypan blue were added to 10–50 μl samples of suspension depending on cell concentration. The dead and live cells (stained and clear, respectively) were counted in Burken cell. Only these suspensions which contained less than 5% of dead cells were taken to the experiment. The final concentration of cells was $3 \cdot 10^6$ per ml. The separation of T and B lymphocytes was accomplished according to the method of Clark and Bost (14).

Immunofluorescence test

5 μl monoclonal antibodies anti lymphocyte T or B labeled with FITC or the same volume of PBS were placed in eppendorff tubes. Subsequently 100 μl of cell suspension was added to the tubes and the samples were incubated on ice for 30 min. Afterwards 1 ml of cold PBS was added and the samples were centrifuged. Pellets were resuspended in 100 μl of PBS. The counts were obtained using flow cytofluorimeter FACSTAR (Becton Dickinson) and the results were analyzed using PC–lysis program (Becton – Dickinson).

Assay of enzymes activity in cell suspensions

The assay of ecto–ATPase activity of intact lymphocytes was conducted according to modified

method of A. Filippini (15). Lymphocytes were washed twice with the working buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 10 mM glucose, 50 mM VO_4^{3-}, 0.1% serum albumin, 20 mM Hepes, and 1 mM sodium azide and subsequently centrifuged. Cell pellets containing $0.5 \cdot 10^6$ cells were added to the above buffer, preincubated at 37°C and the measurements were performed. The reaction was started by the addition of ATP (the final concentration 2 mM). The reaction was terminated by the addition of 10% solution of SDS (sodium dodecyl sulfate). ATPase activity was determined based on the released inorganic phosphate, concentration of which was measured by the Fiske–Subbarow method. The activity was expressed as µmol Pi/10^6 cells/1h.

Cell membrane integrity was determined by trypan blue dye exclusion. In addition, to estimate cell disruption and spill of intracellular enzymes, the activity of cytoplasmic enzyme – lactate dehydrogenase (LDH) – was measured in supernatants of lymphocytes after incubation (16).

No measurable extracellular activities of LDH were found during 1h incubation of lymphocytes B and T indicating the lack of cell breakage. At least 95% viability was required for the experiments reported. The samples with LDH activity below 5% of that in lymphocyte homogenate were qualified for analysis. If the viability was less than 95% contaminating dead cells and cytoplasmic enzymes were successfully removed by a second Ficoll–Hypaque gradient centrifugation (17).

In vitro studies

In vitro evaluation of the influence of some substances on the activity of ecto–ATPase was conducted on „whole lymphocytes'' isolated from the spleen 48 hours after immunization (time required for the most potent immunological response (18)). The separation of lymphocytes into subpopulations was carried out according to the method described above.

The suspensions of lymphocytes were incubated for 30 min in solutions containing LMS, NTG, DTC and FSBA, the final concentrations of which were equal to 10 mM. Following the incubation, the activity of ATPase was determined. At the same time, the activity of LDH was estimated. All substances were dissolved in water, only FSBA in DSMO (the same amounts of DSMO were added to control samples).

RESULTS AND DISCUSSION

The changes in activity of lymphocytes ecto–ATPase under the influence of immunomodulators in rats immunized with SRBC – *in vivo studies*

For our studies on the influence of particular immunomodulators on the cells of immune system we chose one of membrane enzymes – ATPase (3.6.1.3). This enzyme belongs to the group of enzymes integrally bound with cytoplasmatic membrane with catalytic site directed outside of the cell. The enzyme is catalyzing the hydrolysis of extracellular ATP (ATPe) and therefore participates in a number of processes provoked by ATP(e) and its degradation products (19). For the purpose of our experiments we chose three widely used immunomodulators: Levamisole (LSM), diethyldithiocarbamate (DTC) and nitrogranulogen (NTG). The mechanism of action of these substances, from

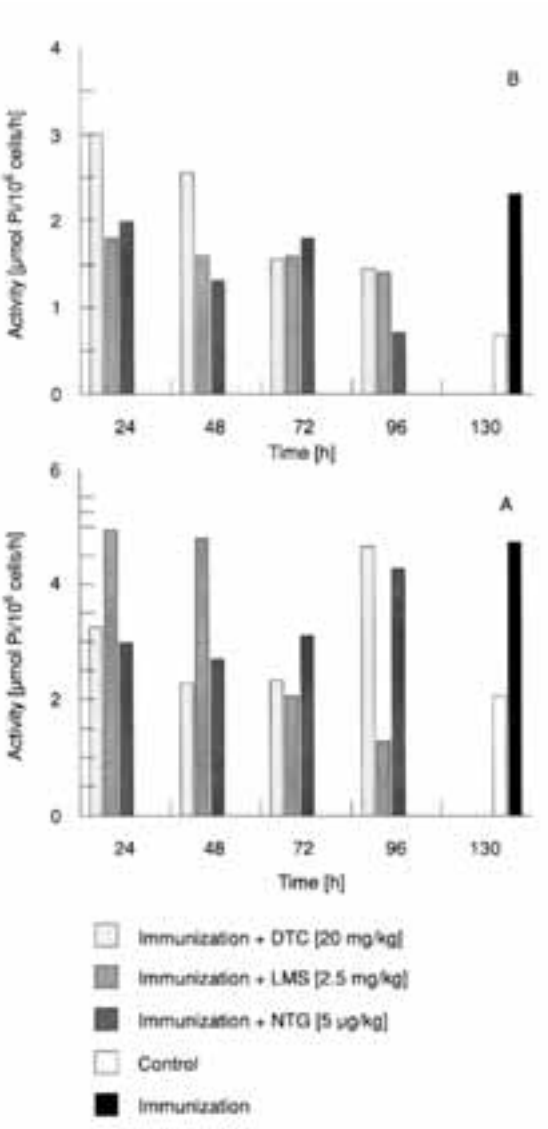


Figure 1.

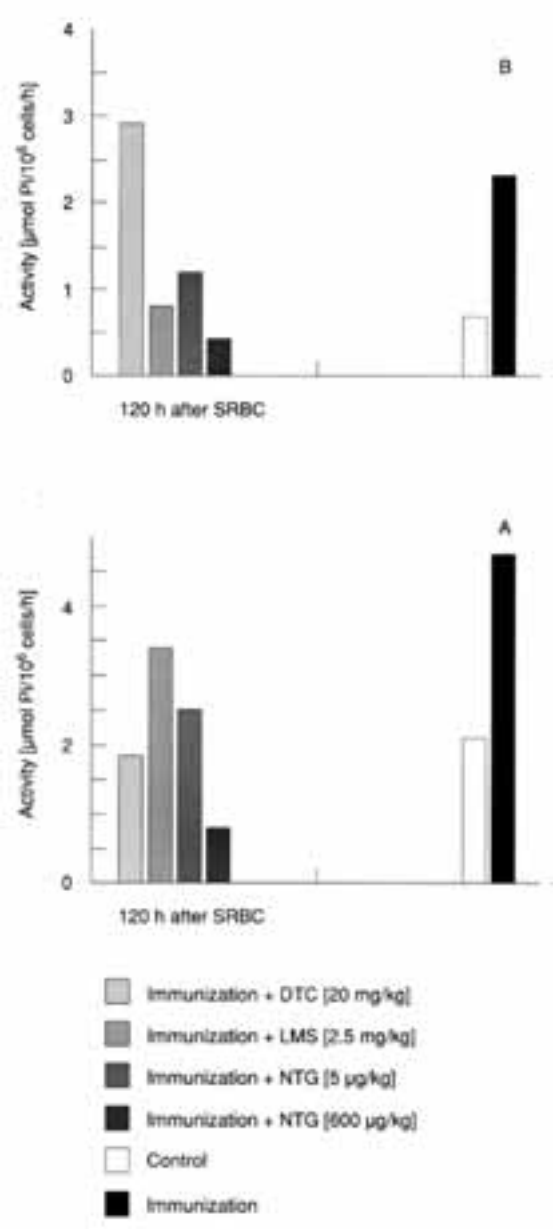


Figure 2.

the point of view of molecular biology, provokes many controversies. In our experiments we used rats immunized by thymus dependent antigen – sheep red blood cells (SRBC). These cells induce humoral response via macrophages and lymphocytes T–helper which activiate lymphocytes B stimulating their proliferation and in consequence the production of antibodies (20). Potentialization of humoral response depends on the dose and the time course of the drug administration. A single or multiple doses of the drug at certain time intervals since immunization may be applied. We are trying to answer the question whether the factors described above influence the function and activity of membrane enzymes of lymphocytes. We monitored the changes of activity of ecto–ATPase in intact lymphocytes B and T using various doses of immunomodulators. During the measurements we maintained the conditions protecting the integrity of the cells membrane. We used a single dose or four doses of the drug. Lymphocytes were collected 120 hours after SRBC immunization. Changes in lymphocytes ecto–ATPase activity after immunization are presented in Figures 1A, 1B, 2A, 2B.

A significant increase in ecto–ATPase activity was observed in both populations of lymphocytes in immunized rats in comparison with the control group. Potentialization of this effect in B lymphocytes was achieved by using a single injection of LMS at 24th hour (Figure 1A). A similar effect was observed in the case of T lymphocytes after a single injection of DTC at 24th and 48th hour after immunization (Figure 1B). Four injections of LMS did not maintain the increased activity of ecto–ATPase from lymphocytes B, however, quadruple doses of DTC kept the elevated activity of the enzyme in lymphocytes T (Figure 2B).

DTC – an immunomodulator restoring the proper ratio of T helper/T suppresser is also known as an agent possessing chelating properties, entering lipophylic complexes with metal ions, easily crossing cellular membranes and influencing activity of cytoplasmatic enzymes, particularly these with –SH group in active site (21,22). The effect of this enzyme on ecto–ATPase from two subpopulations of lymphocytes is markedly different which may suggest differences in the composition of active center of ecto–ATPase in the subpopulations.

Employment of another immunomodulator – NTG – which acts by attacking nucleophilic groups of proteins or nucleic acids (SH–groups among others)(23), causes different response of ecto–ATPase. When cytotoxic dose (600 µg/kg) is administered an evident decline in enzyme activity, below the control value, can be observed (Figures 2A and 2B).

Levamisole – synthetic phenylimidazolthiazol – is mainly used in parasitic infections. It destroys parasites by acting as a selective nicotinic agonist disturbing their ion balance (13). Levamisole due to its immunostimulating properties has been adopted in therapy of diseases connected with impairment of organism immunity such as: chronic inflammations, connective tissue disorders and cancer (13). In enzymology levamisole is known as the inhibitor of membrane alkaline phosphatases (24) and the modulator of Ca^{2+} transporting ATPase

(25). However, the unknown mechanism of its action and contradictory resuls of *in vivo* and *in vitro* studies suggest the necessity of more intensive interest from the point of molecular biology. In our studies levamisole in repeated doses displayed the properties of ecto–ATPase inhibitor for both subpopulations of lymphocytes. In the case of LT a profound decrease in activity was observed approximating the control values (Figures 2A and 2B).

Influence of inhibitors on activity of ecto–ATPase – *in vitro* studies

We conducted the studies on intact lymphocytes collected 48 hours after immunization. Using FSBA, a well known inhibitor of ecto–ATPase of mouse natural killer cells, cytotoxic lymphocytes and human epidermal cells (26,27), resulted in the decrease in enzyme activity to 32% for lymphocytes B and to 28.5 for lymphocytes T (Table 1).

Adding FSBA to the preincubation mixture containing LMS causes inactivation of ecto–ATPase in both subpopulation of lymphocytes. LMS alone did display inhibiting properties *in vitro*. DTC and NTG also turned out to be the inhibitors of ecto–ATPase (Table 1). Adding FSBA to preincubation mixture containing NTG and DTC did not cause further inactivation of enzyme. It might suggest that *in vitro* NTG and DTC modified a part of the enzyme which is attacked by FSBA.

FSBA forms bonds with nucleophilic parts of amino acid residues of catalytic center (26), therefore it might be spectulated that NTG and DTC also attack these sites. It is in agreement with other studies suggesting that NTG and DTC react with thiol groups (–SH) of amino acids. The difference of LMS action suggests other mechanism of its action on ecto–ATPase.

Biochemical consequences of the changes of ecto–ATPase activity

Ecto–ATPase, as an enzyme participating in intracellular metabolism of ATP, cooperates or maybe competes with other enzymes found in cytoplasmatic membrane using $ATP_{(e)}$ as a substrate e.g. apyrases, ATP–pyrophosphatases (27). In the processes regulated by these enzymes also a group of purine receptors is engaged. There are at least two groups of these receptors: P^2 – purine nucleotides with preference to ATP and P^1 – sensitive to adenosine (28). Ecto–ATPase degrading ATP is limiting the time of its effect on P_2 receptor. It seems that apyrase which hydrolyses ATP directly to AMP omitting ADP which has affinity for P_2, is more effective in the liberating of this receptor.

Table 1. Changes in the activity of ecto–ATPase in „intact" lymphocytes isolated from spleen of SRBC immunized rats – *in vitro* studies

	Relative activity (percent of control)	
	Lymphocytes B	Lymphocytes T
Control [a)]	100.0	100.0
10 mM LMS	97.5	98.5
10 mM DTC	49.5	63.0
10 mM NTG	55.5	33.5
10 mM FSBA	32.0	28.5
10 mM LSM + 10 mM FSBA	63.0	53.5
10 mM DTG + 10mM FSBA	51.0	60.0
10 mM NTG + 10mM FSBA	58.5	30.0

[a)] The activity of lymphocytes ecto–ATPase isolated at 48th hour after the immunization

The nature of correlation of ecto–enzymes and puroreceptors still remains an open question. In our studies we observed a significant increase in activity of ecto–ATPase from both subpopulations of lymphocytes in immunization state. Application of DTC and NTG in single doses decreased the activity of the enzyme but not below the control value. Administering multiple doses caused a decrease of activity below the control value (particularly the case of cytotoxic dose of NTG). The decrease of ecto–ATPase activity may prolong the time of $ATP_{(e)}$ influence on the cell membrane – purorceptor P_2 is not liberated. In consequence this can lead to the increase of membrane permeability and intercellular changes (29). It has been observed that in human lymphocytes B a decrease of ecto–ATPase activity causes a sharp increase of intracellular Ca^{2++} entering cytoplasm both from outside and from internal „cisterns" (30,31). It modulates the activity of enzymes dependent on Ca^{2++} and can also induce apoptosis. This phenomenon has been observed in thymocytes, hepatocytes and various lymphocytes cell lines (32). Communication between various lymphocytes plays a particularly important role in immune system. Adenosine (Ado), which is the final product of $ATP_{(e)}$ degradation, is thought to be a contact molecule in the communication between cells. Ado is trapped by P_1 receptors present on human lymphocytes T (but not on lymphocytes B). This causes intracellular changes either through activation of adenyl cyclase or participating of Ado in

nucleotides resynthesis inside the cell (33). Similar phenomena have been observed in the interaction between endothelial cells (Ado generators) and heart muscle cells (possessing Ado receptors)(34). In lymphocytes T of rats penetrated by DTC we observed a different situation. Single doses applied in the period of immunological response potentialization (24 and 48 hours) and multipe doses increased the activity of ecto–ATPase even above the levels in the immunization state. High activity of ecto–ATPase reduces the time of nucleotide action on the cellular membrane but at the same time protects the cells against lytic action of $ATP_{(e)}$. The base of this phenomenon is given by the theory of Filippini (15) which tries to explain the role of ATP(e) and ecto–ATPase in cell mediated cytotoxicity. Murine cytotoxic lymphocytes (CTL), activated for example during an infection, secrete ATP into external medium which participates in cytotoxic attack on target cell. CTL themselves are resistant to the lytic action of $ATP_{(e)}$ due to the high activity of ecto–ATPase. Whether the high activity of that enzyme is sufficient for protection of cell integrity is not clear. The studies on other cells participating in immunological processes do not confirm this role. The current theory of molecular basis of cellular toxicity does not explain in full the role of $ATP_{(e)}$, membrane enzymes and puroreceptors in the interaction between CTL and a target cell.

CONCLUSIONS

1. The induction of immunization state causes the increase in the activity of ecto–ATPase in both subpopulations of lymphocytes B and T.

2. Changes of activity depend on doses and frequency of immunomodulator administration and the effects are different for both subpopulation of lymphocytes.

3. The differences in the results from the studies on animals (*in vivo*) and on isolated lymphocytes (*in vitro*) were obserwed

LMS – in *in vitro* studies does not change the activity of ecto–ATPase but *in vivo* inactivates the enzyme both in single and multiple doses.

DTC – *in vivo* inactivates ecto–ATPase of lymphocytes B both in single and multiple doses. In subpopulation T maintains an increase in activity, especially in multiple doses.

NTG – acts as an inhibitor of ecto–ATPase for both types of lymphocytes.

4. The results obtained suggest that the mechanism of LMS attack on ecto–ATPase is different from that of NTG and DTC action.

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