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PÔVODNÁ PRÁCA *ORIGINAL PAPER*

VPLYV PENICILAMÍNU A AUROTIOJABLČNANU NA HLADINU CYKLICKÉHO ADENOZÍNMONOFOSFÁTU A NA AKTIVITU ADENOZÍNDEAMINÁZY A PURÍNNUKLEOZIDFOSFORYLÁZY V ĽUDSKÝCH T-LYMFOCYTOCH

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THE EFFECT OF PENICILLAMINE AND AUROTHIOMALATE ON CYCLIC ADENOSINE MONOPHOSPHATE LEVEL AND ON ADENOSINE DEAMINASE AND PURINE NUCLEOSIDE PHOSPHORYLASE ACTIVITIES IN HUMAN T-LYMPHOCYTES

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Súhrn

Pozadie problému: **Penicilamín a soli zlata in vitro aj in vivo preukazujú imunosupresívny účinok. Adenozín má imunoregulačné vlastnosti a jeho fyziologická funkcia je v úzkom vzťahu k jeho metabolizmu.**

Cieľ: **In vitro sa sledoval vplyv penicilamínu a aurotiojablčnanu na metabolizmus adenozínu: na aktivitu adenozíndeaminázy a na hodnotu cyklického adenozínmonofosfátu v ľudských lymfocytoch.**

Metodika: **Lymfocyty sa izolovali z heparinizovanej venóznej krvi mladých zdravých darcov na Ficoll-Hypaque hustotnom gradiente. Enzýmové aktivity sa určovali citlivými špecifickými rádiochromatografickými metódami. Hodnota cyklického AMP sa určila technikou RIA.**

Výsledky: **Penicilamín a aurotiojablčnan v závislosti na použitej koncentrácii významne znížili aktivitu adenozíndeaminázy a purínnukleozidfosforylázy a zvýšili hodnotu cyklického AMP v izolovaných bunkách.**

Závery: **Výsledky poukazujú na možný imunosupresívny účinok penicilamínu a aurotiojablčnanu prostredníctvom ovplyvnenia metabolizmu adenozínu v lymfocytoch.**

Kľúčové slová: adenozíndeamináza, aurotiojablčnan, cyklický adenozínmonofosfát, penicilamín, purínnukleozidfosforyláza, T-lymfocyty.

Summary

Background of the problem: **The immunosuppressive effect of penicillamine and gold compounds in vitro as well as in vivo have been demonstrated. Adenosine has been shown to have immunoregulatory properties which are in close relationship to adenosine metabolism.**

The aim of study: **The aim of this study was in vitro investigation of penicillamine and aurothiomalate effects on adenosine metabolism. Therefore adenosine deaminase activity and cyclic adenosine monophosphate level in lymphocytes were examined.**

Methods: **Lymphocytes were isolated from heparinized peripheral venous blood of clinically healthy young men on the Ficoll-Hypaque density gradient. Enzyme activity was determined by sensitive and specific radiochromatographic method. Cyclic AMP level was estimated by RIA assay kit.**

Results: **Penicillamine and aurothiomalate, depending on the concentration used in medium, included a significant decrease of adenosine deaminase activity and an increase of cyclic adenosine monophosphate level in lymphocytes. Both drugs decreased also the activity of another degrading enzyme of purine metabolism — purine nucleoside phosphorylase.**

Conclusion: **This study concluded that the immunosuppressive effect of penicillamine and aurothiomalate could be mediated through the interference of drugs with adenosine metabolism in lymphocytes of patients suffering from rheumatoid arthritis.**

Key words: adenosine deaminase, aurothiomalate, cyclic adenosine monophosphate, penicillamine, purine nucleoside phosphorylase, T-lymphocytes.

ÚVOD

Adenozín preukazuje imunoregulačné vlastnosti: alteruje povrchové antigény a receptory T-lymfocytov (1, 23) a potláča diferenciáciu B-lymfocytov vyvolanú antigénom alebo mitogénom PWM (1). Fyziologická funkcia adenozínu úzko súvisí s jeho metabolizmom. Adenozín sa metabolizuje troma metabolickými dráhami: deamináciou, fosforyláciou a konjugáciou s homocysteínom. Adenozín je substrátom pre adenozíndeaminázu (E.C.3.5.4.6.), ktorá deaminuje adenozín na inozín. Účinkom adenozínkinázy (E.C.2.7.1.20.) z adenozínu vzniká adenozínmonofosfát (AMP). Ďalšou fosforyláciou postupne vzniká adenozíndifosfát (ADP) a adenozíntrifosfát (ATP), z ktorého účinkom adenylátcyklázy vzniká cyklický 3',5'-adenozínmonofosfát (cAMP). Konjugáciou adenozínu s homocysteínom vzniká S-adenozylhomocysteín, ktorý inhibuje metylačné reakcie potrebné pri syntéze RNA a pri proteosyntéze.

Cieľom práce bolo in vitro sledovať vplyv penicilamínu a aurotiojablčnanu na aktivitu adenozíndeaminázy (deaminačná dráha) a na obsah cyklického AMP v lymfocytoch (fosforylačná dráha). Zároveň sa v týchto bunkách sledoval vplyv uvedených liečiv na aktivitu ďalšieho enzýmu degradácie purínov: purínnukleozidfosforylázy (E.C.2.4.2.1.), ktorá katalyzuje premenu inozínu na hypoxantín (15).

MATERIÁL A METODIKA

Chemikálie

Chemicky čistý D-penicilamín (Léčiva Praha), sodná soľ aurotiojablčnanu (Byk Gulden), adenozín, inozín a hypoxantín (Sigma), dextran T500 a Ficoll 400 (Pharmacia), Isopaque (Neygaard), rádioaktívny U-^{14}C-adenozín a 8-^{14}C-inozín a súprava na stanovenie cyklického AMP (Ústav pre výskum, výrobu a využitie rádioizotopov Praha). Ostatné použité chemikálie boli chemickej čistoty p.a. (Lachema Brno).

Izolácia lymfocytov

Lymfocyty sa izolovali z heparinizovanej venóznej krvi mladých zdravých darcov na Ficoll-Hypaque hustotnom gradiente (3).

Stanovenie enzýmových aktivít

Aktivita adenozíndeaminázy (ADA) a purínnukleozidfosforylázy (PNP) sa určovala citlivou a špecifickou rádiochromatografickou metódou podľa Pecháňa (17). Lymfocyty sa 30 min pri 37 ˚C predinkubovali s liečivom alebo s tlmivým roztokom (kontrola). Potom sa 30 min pri 37 ˚C inkubovali s rádioaktívnym substrátom: U-^{14}C-adenozín v Tris-HCl tlmivom roztoku pH 7,4 (ADA) alebo 8-^{14}C-inozín v Tris-HCl tlmivom roztoku pH 7,2 (PNP). Inkubácia sa ukončila ponorením vzoriek do vriaceho vodného kúpeľa

INTRODUCTION

Adenosine has been shown to have immunoregulatory properties which include alteration of T-lymphocyte surface antigens and receptors (1, 23) and the induction of suppressor activity for in vitro pokeweed mitogen and antigen driven B-lymphocyte differentiation (1). The physiological function of adenosine is closely linked to its metabolism. The metabolic fate of adenosine depends on the relative rates of deamination, phosphorylation and conjugation with homocysteine. Adenosine is a substrate for adenosine deaminase (E.C.3.5.4.6.) which deaminates adenosine to inosine. Through the action of adenosine kinase (E.C.2.7.1.20.) the production of adenosine monophosphate is catalysed, then arises ADP and ATP from which cyclic adenosine monophosphate (cAMP) is synthetized by adenylate cyclase. The conjugation of adenosine with homocysteine results in S-adenosyl-homocysteine that inhibits RNA methylation reactions required for RNA and protein synthesis.

The aim of this study was in vitro investigation of the influence of penicillamine and aurothiomalate upon the activity of adenosine deaminase (ADA) and upon cyclic adenosine monophosphate level in lymphocytes. At the same time was determined the activity of another T-lymphocyte enzyme (15): purine nucleoside phosphorylase (E.C.2.4.2.1.).

MATERIALS AND METHODS

Chemicals

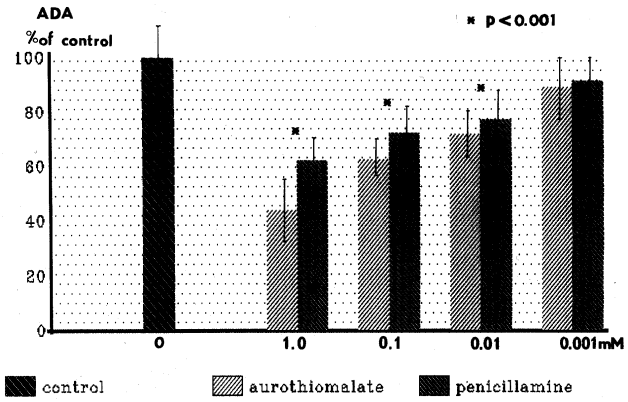
Chemically pure D-penicillamine was gift from Léčiva Prague, sodium aurothiomalate from Byk Gulden. Adenosine, hypoxanthine and inosine were obtained from Sigma. Dextran T500 and Ficoll 400 from Pharmacia, sodium metrizoate (Isopaque) from Neygaard, cyclic AMP assay kit, U-^{14}C-adenosine and 8-^{14}C-inosine from Institute for Research, Production and Application of Radioisotopes Prague. Other materials were used from Lachema Brno.

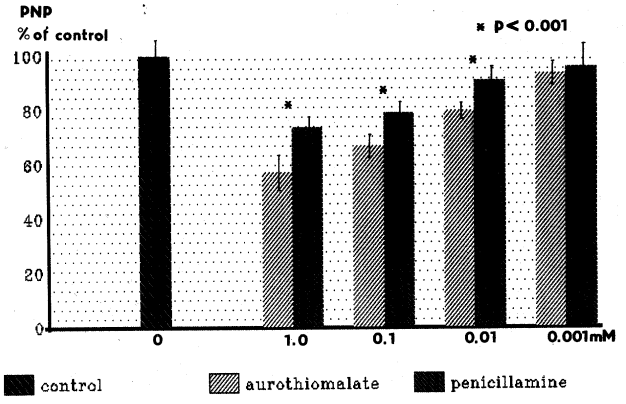
Separation of lymphocytes

Lymphocytes were isolated from heparinized peripheral venous blood of clinically healthy young men on the Ficoll-Hypaque solution at the density of 1.077 kg.l^{-1} (3).

Estimation of enzyme activities

The activity of adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) were estimated by sensitive and specific radiochromatographic methods according to Pecháň et al. (17). Cells were preincubated for 30 min at 37 ˚C with drug or with buffered medium (control) and then were incubated for 30 min at 37 ˚C with radioactive substrate: U-^{14}C-adenosine in Tris-HCl buffer pH 7.4 (ADA) or 8-^{14}C-inosine in Tris-HCl buffer pH 7.2 (PNP). The incubation was termined by dipping of samples into boiling bath





Obr. 1. Vplyv penicilamínu a aurotiojablčnanu na aktivitu adenozíndeaminázy (ADA).

Fig. 1. The effect of penicillamine and aurothiomalate on adenosine deaminase activity.

Obr. 2. Vplyv penicilamínu a aurotiojablčnanu na aktivitu purínnukleozidfosforylázy (PNP).

Fig. 2. The effect of penicillamine and aurothiomalate on purine nucleoside phosphorylase activity.

na 2 min. Po odstredení sa 20 µl supernatantu nanieslo spolu s nerádioaktívnymi štandardnými roztokmi (adenozín, inozín a hypoxantín) na chromatografický papier Whatman 1. Chromatogram sa 18 h vzostupne vyvíjal v zmesi izopropanol—voda—25 % hydroxid amónny v pomere 85:15:1,5 (ADA) a v zmesi nasýtený síran amónny – 0,5 M octan sodný – izopropanol v pomere 160:36:4 (PNP). Oddelené škvrny adenozínu, inozínu a hypoxantínu sa zakreslili pod UV lampou, vystrihli sa a merali na tekutom scintilačnom spektrometri Packard TriCarb. V blankových vzorkách sa substrát v tlmivom roztoku pridal až na konci inkubácie a vzorky sa ihneď ponorili na 2 min do vriaceho vodného kúpeľa.

Stanovenie cyklického adenozínmonofosfátu

Hodnota cyklického AMP sa v lymfocytoch určovala súpravou na jeho stanovenie. Princíp stanovenia spočíva v konkurenčnom viazaní (^{3}H)-cAMP so známou rádioaktivitou a nerádioaktívneho cAMP na špecifickú väzbovú bielkovinu. Na bielkovinu viazaný cAMP možno od neviazaného oddeliť adsorpciou voľného nukleotidu na aktívne uhlie a následným odstredením.

Štatistika

Výsledky sa štatisticky vyhodnocovali Studentovým t-testom. Sú vyjadrené ako aritmetický priemer šiestich vzoriek (každá v troch meraniach)±SD.

VÝSLEDKY

V kontrolnej skupine (100 %) bola v 10^6 lymfocytoch aktivita adenozíndeaminázy 53,50±6,92 pkat a aktivita purínnukleozidfosforylázy 197,03±12,96 pkat.

Predinkubácia lymfocytov s 0,01—1 mM penicilamínom významne znížila aktivitu adenozíndeaminázy (obr. 1).

for 2 min. After centrifugation, 20 µl of supernatant with non-radioactive standard solutions (adenosine, inosine and hypoxanthine) were applied on the chromatographic paper Whatman 1. The chromatogramme was developed ascendently for 18 hours in isopropylalcohol – water – 25 % ammonium hydroxide in the ratio 85:15:1.5, respectively (for ADA) or in saturated ammonium sulphate – 0.5 M sodium acetate – isopropylalcohol in the ratio 160:36:4, respectively (for PNP). The separated spots of adenosine, inosine and hypoxanthine were visualized under ultraviolet light, cut out and counted in a liquid scintillation spectrometer Packard TriCarb. In blank samples, the substrate in buffer was added only after the end of incubation.

Determination of cyclic nucleotide level

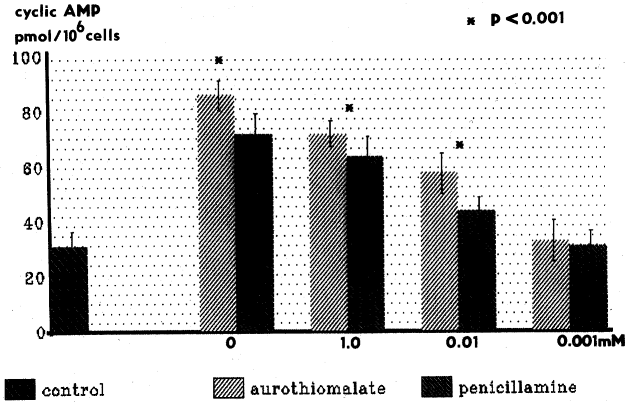
Cyclic adenosine monophosphate level was estimated by cAMP assay kit. Determination is based on the competition between unlabeled cyclic AMP with a fixed quantity of tritium labeled compound for binding to a protein which has a high specificity for cyclic AMP. Separation of the protein-bound cyclic AMP from the unbound nucleotide is achieved by adsorption of the free nucleotide on charcoal, followed by centrifugation.

Statistics

Student's t-test was used for statistical evaluation. Data are expressed as a mean of six samples (in triplicate measurements)±SD.

RESULTS

In control group (100 %) for 10^6 human lymphocytes the enzyme activities of adenosine deaminase and purine



Obr. 3. Vplyv penicilamínu a aurotiojablčnanu na hladinu cyklického adenozínmonofosfátu (cAMP).

Fig. 3. The effect of penicillamine and aurothiomalate on cyclic adenosine monophosphate level.

Liečivo v závislosti od použitej koncentrácie v inkubačnom médiu znížilo aj aktivitu ďalšieho degradačného enzýmu metabolizmu purínov: purínnukleozidfosforylázy (obr. 2). Po predinkubácii buniek s penicilamínom sa v nich zistila zvýšená hladina cyklického AMP (obr. 3).

Podobné účinky preukazoval aj 0,01—1 mM aurotiojablčnan. V izolovaných ľudských lymfocytoch preukazne znížil aktivitu adenozíndeaminázy (obr. 1) aj purínnukleozidfosforylázy (obr. 2) a zvýšil hladinu cyklického adenozínmonofosfátu (obr. 3).

DISKUSIA

Penicilamín aj aurotiojablčnan zasiahli v našom pokusnom usporiadaní do metabolizmu adenozínu, ktorý má imunoregulačné účinky. Obe liečivá znížili aktivitu adenozíndeaminázy (ADA) a purínnukleozidfosforylázy (PNP) a súčasne zvýšili hladinu cyklického AMP v izolovaných lymfocytoch. Stimulovali teda fosforylačnú metabolickú dráhu a inhibovali deaminačnú.

Penicilamín a soli zlata sa už dávnejšie úspešne používajú v liečbe pacientov s reumatoidnou artritídou. Obe liečivá in vitro aj in vivo preukázali imunosupresívne účinky (2, 5, 8, 10, 12). Mechanizmus účinku však zatiaľ nie je dostatočne objasnený.

Blueston a Goldberg (2) zistili, že u pacientov s RA penicilamín významne znížil titer reumatoidného faktora. Počas liečby výrazne klesli aj hladiny IgG a IgM, hladina IgA sa však nezmenila (2). U týchto pacientov zistili vysokovýznamnú koreláciu medzi poklesom hladiny IgM v sére a poklesom titra reumatoidného faktora (2). Iní autori zistili u pacientov s RA po liečbe penicilamínom pokles cirkulujúcich nucleoside phosphorylase were 53.50±6.92 pkat and 197.03±12.96 pkat, respectively.

Pretreatment of lymphocytes with penicillamine in the concentrations 0.01–1mM resulted in significant inhibition of adenosine deaminase activity (Fig. 1). Drug, depending on the concentration used in medium, induced also a decrease of purine nucleoside phosphorylase activity (Fig. 2) and an increase of cyclic adenosine monophosphate level in these cells (Fig. 3).

From Fig. 1 and 2 it is evident that aurothiomalate at the concentrations 0.01—1 mM effectively inhibited the activity of both purine degradative enzymes: adenosine deaminase as well as purine nucleoside phosphorylase. Decrease of ADA and PNP activity was dose dependent. At the same time aurothiomalate significantly elevated cyclic AMP level in isolated lymphocytes (Fig. 3).

DISCUSSION

These data suggest an interference of penicillamine and aurothiomalate with the metabolism of adenosine which has been shown to have immunoregulatory properties. Both drugs inhibited the pathway of deamination and stimulated the pathway of phosphorylation. This fact was manifested by a decrease of ADA and PNP activity and at the same time by an increase in cAMP level.

For a long time penicillamine and gold compounds have been effectively used in the treatment of patients suffering from rheumatoid arthritis (RA). The immunosuppressive effects of both drugs in vitro and in vivo have been demonstrated. The mechanism of their effect has not, however, been fully explained.

Penicillamine-treated RA patients showed a significant reduction in rheumatoid factor titre as shown by the latex-agglutination test (2). Bluestone and Goldberg demonstrated a decrease in serum IgG and IgM levels, however, no effect was observed on IgA level (2). There was a highly significant correlation between the decline in serum IgM level and the fall in latex titre after penicillamine therapy in RA (2). Jaffe described an inhibitory effect of the drug on the level of circulating immune complexes in patients with RA (8). Experiments by Schumacher et al. looked for the suppressive effect of the drug on humoral and cellular response in mice (20, 21). In vitro, penicillamine suppressed by antigens or by mitogens induced stimulation of mouse lymphocytes (10, 11) and lymphocyte transformation in cultures (19). Helperic T-lymphocytes were especially sensitive to the inhibition by penicillamine (12). Any effect in vitro occured on B-lymphocytes. Lipsky and Ziff assumed a selective suppressive effect of penicillamine on T-lymphocyte function (12).

imunokomplexov (8). Schumacher a spol. (20,21) opísali u myší supresívny účinok liečiva na humorálnu aj bunkami sprostredkovanú imunitnú odpoveď. In vitro penicilamín potláča antigénmi a mitogénmi vyvolanú stimuláciu myších lymfocytov (10, 11) a transformáciu lymfocytov v kultúrach (19). Liečivo má špecifický supresívny účinok na helperové T-lymfocyty (12). Na B-lymfocyty nemalo žiadny vplyv (12).

Viacerí autori zistili, že aj soli zlata majú imunosupresívne účinky. U pacientov s RA chryzoterapia znížila v sére hladinu imunoglobulínov aj titer reumatoidného faktora (5). Počas terapie u nich zistili zníženú odpoveď lymfocytov na nešpecifickú stimuláciu mitogénom PWM, ktorý je T-dependentným polyklonovým aktivátorom B-lymfocytov (5). In vitro soli zlata potlačili antigénmi alebo nešpecifickými mitogénmi stimulovanú proliferáciu T-lymfocytov (9, 10).

Zatiaľ nie sú žiadne údaje literatúry o vplyve penicilamínu a aurotiojablčnanu na metabolizmus adenozínu, ktorý má významné postavenie v imunoregulácii. V našich pokusoch sme sa preto zamerali na sledovanie vplyvu týchto liečiv na aktivitu adenozíndeaminázy a purínnukleozidfosforylázy a na hladinu cyklického AMP v lymfocytoch.

Parker a spol. (16) už dávnejšie zistili, že cAMP je intracelulárnym mediátorom stimulácie lymfocytov antigénmi a mitogénmi. Adenozín zvyšuje hladinu cAMP tak, že interaguje so špecifickými receptormi lymfocytov, a tým aktivuje adenylátcyklázu (13), ktorá katalyzuje premenu ATP na cAMP. Iným mechanizmom potlačenia odpovede lymfocytov je zvýšenie hladiny cAMP inhibovaním jeho degradácie prostredníctvom fosfodiesterázy (6).

Aktivita adenozíndeaminázy je potrebná na normálnu funkciu lymfocytov (4, 7, 22). Tento enzým je potrebný na stimuláciu ľudských aj myších lymfocytov mitogénmi (7). Adenozíndeamináza tvorí s ektoenzýmom dipeptidylpeptidáza aktivačný antigén (CD26) T-lymfocytov (14). V zhode s týmito pozorovaniami majú jedinci s vrodenou deficienciou adenozíndeaminázy významne zníženú humorálnu aj bunkami sprostredkovanú imunitu (18). Aj vrodená deficiencia purínnukleozidfosforylázy je spojená s narušením celulárnej imunity (18, 24).

Z uvedených pozorovaní rôznych autorov je zrejmé, že zvýšená hladina cyklického AMP a znížená aktivita adenozíndeaminázy a purínnukleozidfosforylázy sú sprevádzané potlačenou odpoveďou ľudských aj myších lymfocytov.

V našom pokusnom usporiadaní penicilamín a aurotiojablčnan znížili aktivitu adenozíndeaminázy aj purínnukleozidfosforylázy a zvýšili hladinu cyklického AMP. Na základe dosiahnutých výsledkov predpokladáme, že imunosupresívny účinok penicilamínu a aurotiojablčnanu môže byť u pacientov s reumatoidnou artritídou spôsobený ich zásahom do metabolizmu adenozínu v lymfocytoch.*

*Autori ďakujú p. S. Boháčovej za technickú spoluprácu.

Several authors have already described the immunosuppressive effect of gold compounds. In patients suffering from RA chrysotherapy suppressed serum immunoglobulin level and rheumatoid factor titre (5). In RA patients receiving gold therapy, there was also observed a suppressed response of lymphocytes to non-specific stimulation by pokeweed mitogen which is considered to be a T-dependent polyclonal activator of B-lymphocytes (5). In vitro gold salts suppressed by antigens or by non-specific mitogens stimulated proliferation of T-lymphocytes (9, 10).

Till now there is no evidence about the effect of penicillamine and aurothiomalate on adenosine metabolism which is in close relationship to immunoregulation. Therefore, we have studied the effect of these drugs on cyclic AMP level and on the activity of adenosine deaminase and purine nucleoside phosphorylase in isolated human lymphocytes.

The data of Parker et al. (16) indicate that cyclic AMP is involved as an intracellular mediator of lymphocyte stimulation by antigens or mitogens. Adenosine is capable of increasing cAMP level in lymphocyte as a consequence of its interaction with a specific membrane receptor, which results in the activation of adenylate cyclase (13). Another mechanism of lymphocyte response suppression is an increase in cAMP production as a result of cAMP phosphodiesterase inhibition (6).

Adenosine deaminase activity is particularly important for normal lymphocyte function (4, 7, 22). The activity of this enzyme is essential for stimulation of human and mouse lymphocytes by mitogens (7). Adenosine deaminase on the surface T-lymphocytes binds to the activation antigen CD26 which is identical with ectoenzyme dipeptidylpeptidase IV (14). In agreement with these observations, individuals with inherited ADA deficiency suffer from severe suppression of both humoral and cell-mediated immunity (18). Decreased purine nucleoside phosphorylase activity is also associated with disturbance of cell-mediated immunity (18, 24).

From previous observations it is evident that increased cAMP level and decreased ADA and PNP activities are accompanied by suppressed response of human lymphocytes. In our experiments we have looked for the effect of penicillamine and aurothiomalate on adenosine metabolism which is in close relationship to immunoregulation. Both drugs increased cyclic AMP level and decreased ADA and PNP activity. The latter fact refers to the immunosuppressive effect of the tested drugs.

Our study concluded that the immunosuppressive effect of penicillamine and aurothiomalate could be mediated through the interference of the drug with adenosine metabolism in the lymphocytes of patients suffering from rheumatoid arthritis.*

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