in the phenol ring enhances the antiinflammatory activity. Elimination of a strong polarized carbonyl group boosts the lipoidotropy of a compound, intensifying its antiinflammatory activity. The presence of a 15-methyl group in the 2,3-dimethoxy-substituted diazasteroid causes the efficacy to decline, according to a number of tests. Hormonal activity is not inherent to 8,16-diazagonans VI and VII, which impinge upon many elements of the inflammatory process, whereas unsubstituted diazasteroid I is found to boost glycogen deposition in rat liver. The results of this investigation hold promise of a fruitful search for antiinflammatory substances in the 8,16-diazasteroid series that do not have any perceptible hormonal activity and that differ both from corticosteroids and from nonsteroid antiinflammatory agents.

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Effect of Cortisol and Pertussis Toxin on the cAMP Concentration in Human Lymphocytes

A. S. Dukhanin, L. I. Stankevich, and M. P. Petrova

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It is demonstrated that pertussis toxin and hydrocortisone potentiate the adenosine-induced rise of the cAMP concentration in lymphocytes. Hydrocortisone elicits an immediate (for the simultaneous addition of adenosine and cortisol) and reversible effect. The effect of pertussis toxin has a latency and is irreversible. Added together, these agents exert no cumulative effect. It is assumed that hydrocortisone and pertussis toxin have the same target - the inhibiting regulatory protein G_i.

Key Words: *cortisol; pertussis toxin; adenosine; cAMP; lymphocytes*

Molecular aspects of corticoid-dependent modulations of the second messenger levels in competent cells have hardly been studied. It has been assumed that glucocorticoid hormones potentiate the effects of adenylate cyclase activators (β-adrenoagonists, adenosine, and prostaglandin E_2) by increasing the coupling of receptor and catalytic subunits of the adenylate cyclase system [5,6].

Department of Molecular Pharmacology and Radiobiology, Russian State Medical University, Moscow. (Presented by P. V. Sergeev, Member of the Russian Academy of Medical Sciences)

Peripheral blood lymphocytes are a convenient model, since their biological response to glucocorticoids has been studied in sufficient detail and is easy to reproduce. They have two types of plasma membrane receptors for adenosine: A_1 and A_2 [2,3]. The interaction between A_2 receptors and adenosine increases the activity of adenylate cyclase and elevates the intracellular cAMP content. The A_1 receptors are coupled to the enzyme via the regulatory G_i protein, which mediates the inhibitory effects of various hormones and neurotransmitters on adenylate cyclase activity [8]. Therefore,

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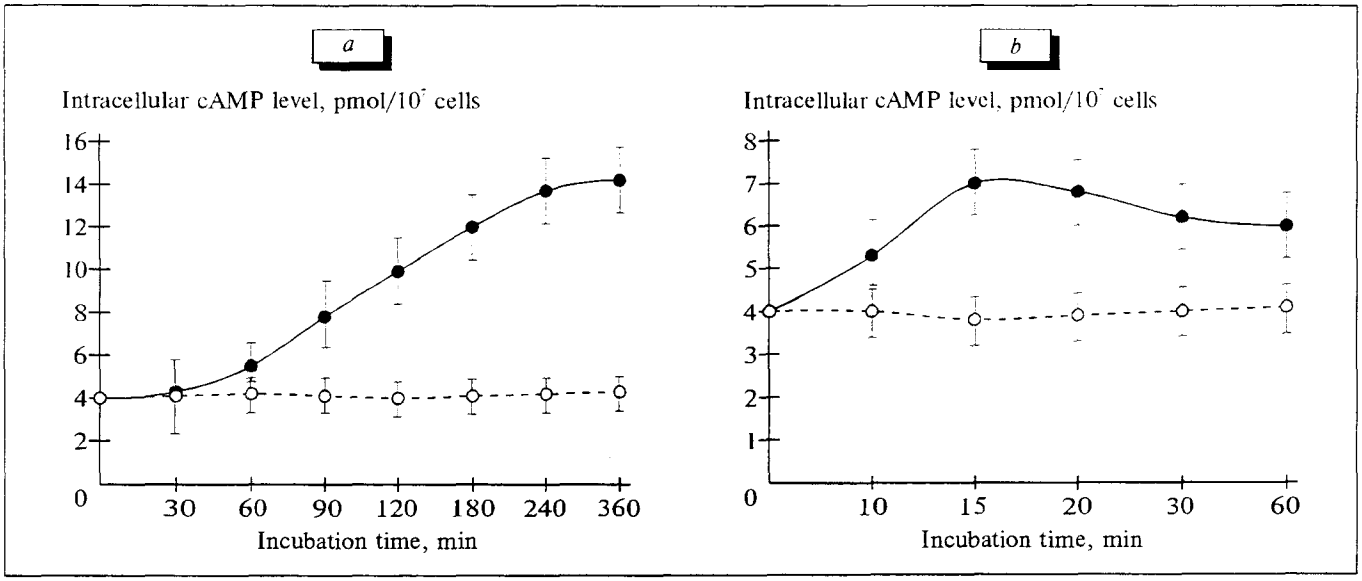

Fig. 1. Effect of PT (*a*, 0.33 μg/ml) and HC (*b*, 3 μM) on the cAMP content in lymphocytes. Broken line shows the baseline cAMP level.

the A_1 type receptors mediate the decrease in the intracellular cAMP content. The integral cell response to adenosine is determined by the A_1/A_2-receptor ratio, the concentration of these receptors, and their affinity for the ligand, which are modulated by various factors.

Bacterial toxins have found wide application in experimental practice as a molecular tool regulating the activity of the adenylate cyclase system. Pertussis toxin (PT) exhibits enzymatic activity and catalyzes ADP ribosylation of the α-subunit of G_i protein [4]. As a result of covalent modification of G_i protein, transmembrane transduction of the signal to A_1 receptors is impaired.

The aim of this study was to compare the effects of PT and hydrocortisone (HC) on the basal and adenosine-stimulated cAMP levels in human peripheral blood lymphocytes.

MATERIALS AND METHODS

Lymphocytes were isolated from peripheral blood of healthy donors. Blood (30 ml) was collected in plastic vials containing 6 ml anticoagulant (25 g bihydrate sodium citrate). Blood (5 ml) was layered onto an Isopak-Ficoll gradient (3 ml) and centrifuged at 1500 *g* for 15 min at room temperature. The transparent layer over the gradient was aspirated. Mononuclear leukocytes were resuspended in Hanks medium and washed by centrifugation [1]. The suspension of mononuclear lymphocytes obtained by the method of Boyum (1968) contains 90% lymphocytes and 10% monocytes. The majority of the mononuclear cells are represented by T cells (about 70%).

The test agents (20 ml) or Hanks salts (20 μl, control) were added to the cell suspension (1 μl) after a 40-min incubation. The cell concentration was 10^7 cells/ml (Hanks salts, pH 7.4). The effects of adenosine (a final concentration of 10^{-7} M), HC (1-3 μM), and PT (0.33 μg/ml) on the intracellular cAMP concentration as a function of the incubation time were studied. After the incubation, the cells were lysed in ice-cold Tris-EDTA buffer (pH 7.5), the smaples were immediately immersed in a water bath at 90°C for 3 min, and then centrifuged (10 min at 800 *g*), and the cAMP concentration was determined in the supernatant with the use of the standard Amersham kits.

Statistical analysis, calculation of reliability intervals, and evaluation of the significance of differences were performed using Student's *t* test at the significance level $p=0.05$.

The following reagents were used: adenosine and HC (Serva), Histopaque-1077, Tris, and EDTA (Sigma), and PT (Ufa Chemico-Pharmaceutical Plant, Russia).

RESULTS

Pertussis toxin (0.33 μg/ml) significantly increased the baseline cAMP content in lymphocytes (4.1 pmol/10^7 cells) after a 1-h latent period (Fig. 1, *a*). The cAMP content increased 2.5- and 3.5-fold after 2 and 4 h of incubation with PT, respectively. These results are consistent with the observations of others and indicate that the PT preparation used in this study has a high biological activity [10].

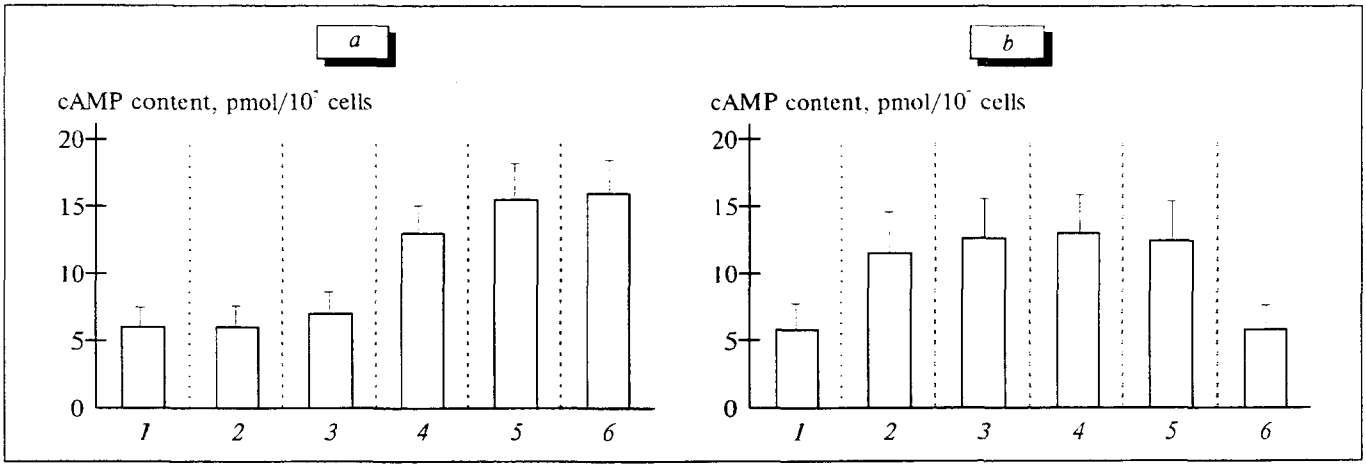

Fig. 2. Effect of PT (*a*) and HC (*b*) on the adenosine–stimulated cAMP increase in lymphocytes. *1*) adenosine (0.1 μM), *a*: 2–5) adenosine + PT (0.33 μg/ml), incubation with PT: 30 min (*2*), 1 (*3*), 1.5 (*4*), and 2 h (*5*). Adenosine was added 10 min before the end of incubation with PT. *6*) cell response to adenosine (0.1 μM) after a 2–h incubation with PT and washing. *b*: 2–5) adenosine + HC (3 μM) without incubation (*2*) and after incubation with HC for 10 (*3*), 20 (*4*), and 30 (*5*) min. *6*) cell response to adenosine (0.1 μM) after a 20–min incubation with HC and washing.

Figure 1, *b* illustrates the dependence of the intracellular cAMP content on the time of incubation of lymphocytes with HC (3 μM). The hormone raised the cAMP level, which reached a maximum by the 15th min of incubation. Further incubation lowered the cAMP concentration, although at the 30th and 60th min of incubation this still remained higher than the baseline level. However, the hormone-induced increase in the cAMP concentration was much less pronounced compared with the PT-induced alterations in the intracellular cAMP content, and the duration of latency was less than 10 min.

In order to elucidate the possible mechanism of HC action on the adenylate cyclase system we compared the effects of PT and glucocorticoids on the adenosine-stimulated rise of the cAMP content in lymphocytes. The effects of PT and glucocorticoids on the adenosine-mediated cAMP increase are shown in Fig. 2. The final concentration of adenosine in the samples was 0.1 μM, which corresponds to the physiological blood level of this purine [7]. It can be seen from the figure that PT and HC significantly stimulate the adenosine-dependent rise of the cAMP level in lymphocytes. At the same time, the potentiating effect of HC, unlike that of PT, was observed immediately after the simultaneous addition of adenosine and cortisol and was reversible. After the cells had been washed and placed in glucocorticoid-free medium, the effect of HC was abolished independently of the time of incubation with the hormone.

Analysis of these results allows one to regard the potentiating effect of glucocorticoids on the adenosine-stimulated increase in the lymphocyte cAMP content as a permissive ("resolving") action of corticosteroids, i.e., a hormonal action which is not mediated by protein synthesis (the so-called "fast" effects of glucocorticoids).

The effect of PT on the cAMP response of lymphocytes proved to have a latent period and to be irreversible (Fig. 2, *a*). In this case the increase in the intracellular cAMP content is explained by the abolition of the inhibitory effect of adenosine mediated by A_1 receptors.

We also studied the combined effect of PT and HC on the cAMP content in lymphocytes. For this purpose the cell suspension was incubated for 1.5 h (the time it takes for development of the PT effect) with PT and HC alone and in the presence of both agents. After the incubation, the lymphocytes were washed, and their response to adenosine was assessed. The cAMP content in the samples containing only PT or HC was 12.5±1.1 and 5.9±0.6 pmol/10^7 cells, respectively. The cAMP content in samples incubated with PT and HC together was significantly lower: 8.7±0.9 pmol/10^7 cells. These findings indicate that during the interval studied, HC prevents the effect of PT on G_i protein of the adenylate cyclase system in lymphocytes.

It can be assumed that HC and PT have the same target: the inhibiting regulatory protein. By inhibiting the activity of G_i protein, HC impairs the coupling of A_1 receptors to adenylate cyclase. These receptors on the lymphocyte plasma membrane become "silent," which shifts the A_1/A_2 functional ratio toward the A_2 subtype.

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Nonspecific Leukocytolysis *In Vitro* as a Test of Comparative Cytotoxicity of Low-Molecular Nonelectrolytes

M. P. Vavilov, A. P. Vorotyntsev, E. A. Luzhnikov, and E. K. Nazarova

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In experiments with hypo-, iso-, and hypertonic solutions taken at equimolar concentrations for equilibrium of osmotic conditions the leukocytolytic action of each of three nonelectrolytes was shown to be a parabolic function of osmotic concentrations. The comparative degree of leukocytolysis for urea is superior to that for glycerin and glucose and in inverse proportion to their molecular weight. As this difference depends on the chemical component of action, nonspecific leukocytolysis *in vitro* is considered as a test of comparative cytotoxicity in contrast to the specific (immunologic) cytotoxicity.

Key Words: *leukolysis; nonelectrolytes; osmosis; cytotoxicity*

At present two methods are generally used for the study of leukocytes as a biological object. The first approach, involving cell count in counting chambers and morphological examination of blood cells [7], serves a diagnostic purpose and is used for assessment of reactivity (for example, the leukocytic index of intoxication) [9] as well as of adaptive reactions of the organism [5]. The second includes a group of techniques among which immunologic assays are used [1,3,8,11] to study the leukocytic response to *the in vitro* action of various infectious and noninfectious agents, as well as to determine specific cytotoxicity [8,11]. The reaction of leukolysis is specific and has been recommended as a tool for comparative study in cases of chemical and drug standardization with regard for their allergenic action [3]. However, these recommendations are hardly implemented due to procedural difficulties, which have been described as follows: "so far, purely osmotic phenomena of irritation are almost impossible to distinguish from purely chemical phenomena" [10]. Nevertheless, this problem (of comparing the nonspecific cytotoxicity of chemical substances) may be partially solved by drawing on a classic proposition of chemistry, namely, that equimolar solutions of nonelectrolytes (NE) of low molecular weight have equal osmotic pressure [6].

The aim of the present investigation was to analyze the quantitative changes and qualitative indexes of nonspecific leukolysis *in vitro* and to validate the leukocytolysis phenomenon as a test of

Department of Clinical Toxicology, Russian Medical Academy of Postgraduate Education, Moscow

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