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REDUCED RESPONSIVENESS OF ADENYLATE CYCLASE TO FORSKOLIN IN HUMAN LYMPHOMA CELLS

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Abstract—The β_2-adrenergic transmembrane signal transduction was investigated in malignant B-cells from 15 patients with low grade non-Hodgkin's lymphoma as compared with normal lymphocytes of seven healthy adults. The number of β_2-adrenoceptors and the response of adenylate cyclase (AC) to isoproterenol were slightly decreased in lymphoma cells. The responsiveness of AC to forskolin was 8-fold lower in lymphoma cells, whereas the response to cholera toxin showed no difference. These findings demonstrate an impairment of the β_2-adrenergic signal transduction in low grade lymphoma cells that particularly affects the function of AC. The comparison with forskolin resistant mutants of an adrenocortical tumor cell line, Y1 (Schimmer *et al.*, *J Biol Chem* **262**: 15521–15526, 1987), suggests that the availability of functional active alpha subunits of stimulatory G proteins (G_s) might be reduced in human B-cell lymphoma, although other mechanisms known to inhibit the AC activity might be involved.

Normal human lymphocytes bear β_2-adrenergic binding sites [1], whose interaction with catecholamines results in the stimulation of the enzyme adenylate cyclase (AC§) via the stimulating GTP binding protein (G_s) [2], thereby increasing the intracellular concentration of cyclic 3′:5′-adenosine monophosphate (cAMP). This second messenger seems to inhibit most functions of mature human lymphocytes including mitogen-induced DNA synthesis [3, 4], anaerobic glycolysis [5], production of lymphokines [6] and antibodies [7], T-cell mediated cytolysis [8–10] and motility of T- and B-cells [11]. However, cAMP has been found to enhance the differentiation of both immature lymphocytes [12] and lymphoma cells [13]. The plasma, urine and intracellular cAMP levels were reported to be decreased in patients with acute (ALL) and chronic lymphocytic leukemia (CLL) [14–17]. Both adrenergic binding sites and the stimulation of AC by catecholamines were shown to be reduced in CLL lymphocytes [18–21]. Recently, the adenylate cyclase system has received increasing attention because of its possible role in malignant transformation [22–29] cAMP was demonstrated to suppress neoplastic B-cell proliferation in human and murine lymphoma cell lines [30, 31]. We therefore conducted a study to determine the number and affinity of β_2-adrenoceptors on normal and malignant human lymphocytes and to investigate the function of the β_2-adrenergic signal transduction (receptor, G_s, AC) in these cells by stimulation with different agents (isoproterenol, cholera toxin and forskolin).

MATERIALS AND METHODS

Patients. Fifteen patients with leukemic low grade non-Hodgkin lymphoma (lymphocyte count $>10^4$ cells/μL) aged from 48 to 79 years (median 68 years; mean ± SD 63.5 ± 10.5 years; seven female eight male) treated in the Medizinische Klinik, Klinikum Innenstadt, Munich, Germany, entered the study. Six apparently healthy subjects and one patient with coronary heart disease without further complications or treatment, aged from 24 to 78 years (median 33; mean ± SD 42.8 ± 20.7; two female, 5 male) served as controls. The histopathological entities according to the Kiel classification [32] were distributed as follows: 12 CLL, two immunocytoma (IC) and one centrocytic–centroblastic lymphoma (cccb). Immunophenotyping of mononuclear leucocytes (MNL) in patients showed that all were B-cell lymphoma, and that more than 90% of MNL were B-cells. CLL and IC patients were staged according to the Binet classification [33], the cccb patient according to the Ann Arbor classification [34]. Three of the 14 CLL and IC patients were in Binet stage A, six in stage B and five in stage C. The cccb patient was in stage IV. No patient received chemotherapy or took sympatholytic or sympathomimetic drugs.

Materials. Hepes buffered Hank's salt solution (HH) consisted of the following compounds diluted

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§ Abbreviations: AC, adenylate cyclase; G_s, stimulatory GTP binding protein; G_i, inhibitory GTP binding protein; cAMP, cyclic 3′:5′-adenosine monophosphate; DNA, deoxyribonucleic acid; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; IC, immunocytoma; cccb, centrocytic–centroblastic; HH, Hepes buffered Hank's salt solution; IBMX, 3-isobutyl-1-methylxanthine; ^{125}ICYP, ^{125}iodo-cyanopindolol; MNL, mononuclear leucocytes; TB, total binding; UB, unspecific binding; K_d, dissociation constant; NaF, sodium fluoride, Gpp(NH)p, guanyl-5′-yl imidophosphate; ACTH, adrenocorticotropic hormone.

in distilled water: 1.26 mmol/L $CaCl_2$, 5.4 mmol/L KCl, 0.44 mmol/L KH_2PO_4, 0.8 mmol/L $MgSO_4$, 136.9 mmol/L NaCl, 0.42 mmol/L Na_2HPO_4 (all salts purchased in analytical purity from Merck, Darmstadt, Germany), and 25.0 mmol/L Hepes (Sigma Chemical Co., St Louis, MO, U.S.A.). This buffer was adjusted to pH 7.4 with 1 N NaOH solution. Forskolin, cholera toxin (vials of 1 mg lyophilized protein), 3-isobutyl-1-methylxanthine (IBMX) and (−)isoproterenol bitartrate were purchased from Sigma, (−)^{125}iodo-cyanopindolol (^{125}ICYP; 2000 μCi/mmol) from Amersham (Braunschweig, Germany), and heparin from Novo Industrie (Mainz, Germany). (−)Timolol maleate was a generous gift from Merck Sharp and Dohme (Munich, Germany).

Binding assay. The experimental procedure was performed as described previously [35], with small modifications. Briefly, samples of 15–20 mL heparinized, venous blood were obtained between 9 and 10 a.m. MNL were harvested by density gradient centrifugation [36]. The MNL fraction was washed twice in HH pH 7.4 and then resuspended in this buffer. Intact cells (5×10^5) were incubated in 100 μL with varying concentrations (1.0–180 pmol/L) of ^{125}ICYP for 120 min at 37° to determine total binding (TB). Unspecific binding (UB) was determined in parallel incubations with 1 μmol/L unlabelled (−)timolol. Specific binding was defined as TB − UB. After the incubation period, the samples were centrifuged at 15,000 *g* for 5 min and the supernatant was removed by suction. The pellets were counted in a Packard Gamma Spectrometer (model 5360).

Specific binding data were iteratively fitted by non-linear regression analysis to a model of two independent binding sites as described earlier [35, 37], using the Enzfitter program from Biosoft®, Cambridge, U.K. This model was used because previous studies in our own laboratory, as well as in other laboratories, suggested the existence of two rather than one binding site for β_2-adrenoceptors on human MNL [35, 37, 38]. Binding data were also plotted according to the method of Scatchard [39] in order to visualize results. Since the functional and molecular properties of the low affinity binding site are yet to be defined, only the results for the high affinity binding site were considered, and the high affinity binding site was called β_2-adrenoceptor. K_d and B_{max} represented the equilibrium dissociation constant and density of the high affinity binding site in the saturation experiments.

Stimulation of AC and cAMP assay. MNL (5×10^5 in 500 μL) were preincubated for 30 min at 20° in the presence of 10^{-3} mol/L IBMX to inhibit phosphodiesterase activity. Thereafter, the cells were incubated at 37° with isoproterenol (0, 10^{-8}, 5×10^{-7} and 10^{-4} mol/L) for 10 min, cholera toxin (0, 10^{-6} and 10^{-4} g/mL) for 60 or 120 min, or forskolin (10^{-4} mol/L) for 15 min. Isoproterenol was diluted in HH buffer, cholera toxin in distilled water, and forskolin in 100% ethanol (control experiments with ethanol revealed no influence on cAMP accumulation). The reaction was stopped by incubating the cells at 100° for 5 min. Thereafter, the cells were centrifuged at 15,000 *g*, 300 μL of the supernatant were removed and stored at −30°, until the cAMP content was determined in duplicate with a [^{125}I]cAMP radioimmunoassay kit of Amersham Braunschweig, Germany [40].

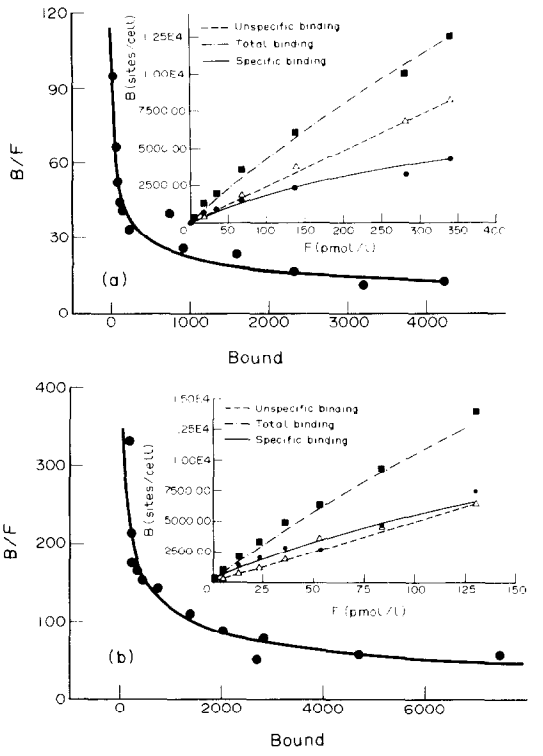

Fig. 1. (a) Binding curves and Scatchard plot of a saturation experiment with ^{125}ICYP and intact lymphocytes of a healthy male adult (78 years old). The binding parameters for the high affinity binding site obtained by non-linear regression analysis were: B_{max} = 455.8 sites/cell, K_d = 1.8 pmol/L. (b) Binding curves and Scatchard plot of a saturation experiment with ^{125}ICYP and intact lymphocytes of a female CLL patient, Binet stage A (75 years old). The binding parameters for the high affinity binding site obtained by non-linear regression analysis were: B_{max} = 74.8 sites/cell, K_d = 0.6 pmol/L.

Statistics. Patients and controls were compared by the Mann–Whitney test for independent samples. A P value <0.05 was regarded as statistically significant. All results are given as mean ± SEM.

RESULTS

β_2-Adrenoceptors in normal lymphocytes and lymphoma cells

Representative experiments of ^{125}ICYP binding to intact MNL are shown in Fig. 1a and b. The number of high affinity ^{125}ICYP binding sites (β_2-adrenoceptors) per cell was 455.8 in MNL of a 74-year-old healthy person (Fig. 1a) and 74.8 in MNL of a 75-year-old CLL patient (Fig. 1b). As a group phenomenon, the number of high affinity β_2-adrenergic binding sites per cell was approximately 3-fold higher in normal lymphocytes (869.0 ± 149.1) than in lymphoma cells (322.8 ± 77.3; P < 0.01).

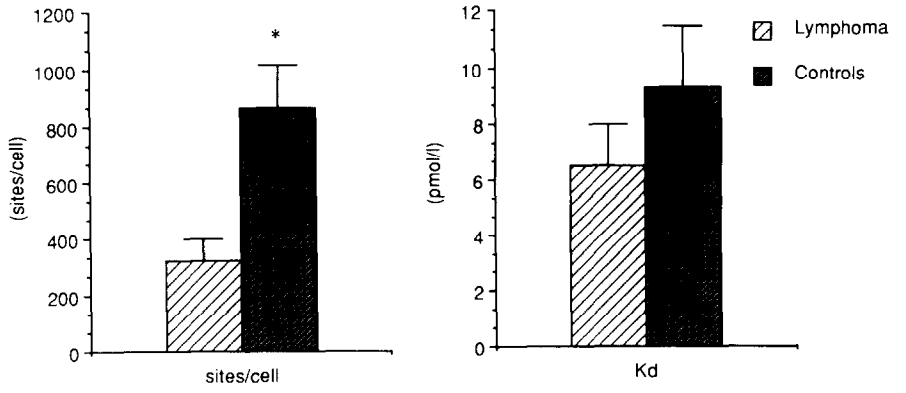

Fig. 2. Density (sites/cell = B_{max}) and equilibrium dissociation constant (K_d) (means ± SEM) of high affinity β_2-adrenergic binding sites of human lymphoma cells (N = 15) and normal lymphocytes (N = 7). * Difference from lymphoma: $P < 0.01$.

The apparent affinity constant (K_d) exhibited no significant difference (Fig. 2).

Stimulation of AC by different agents

The basal cAMP accumulation of normal lymphocytes and lymphoma cells showed no difference and did not significantly change within the incubation periods used (10–120 min). The intracellular cAMP accumulation after incubation with 10^{-4} mol/L isoproterenol was slightly lower in lymphoma cells (12.0 ± 2.0 pmol/10^6 cells) as compared with normal lymphocytes (18.9 ± 4.4 pmol/10^6 cells; $P = 0.06$). At lower concentrations of isoproterenol, the difference in cAMP accumulation between normal and malignant lymphocytes decreased. Cholera toxin increased the intracellular cAMP levels in normal and malignant lymphocytes to the same extent (for all concentrations and incubation periods used); these results, however, showed a considerable interindividual variation, particularly in lymphoma patients as demonstrated by large SEM ranges. Forskolin increased the intracellular cAMP accumulation of lymphoma cells to 22.3 ± 3.3 pmol/10^6 cells (Fig. 3). In normal lymphocytes, this increase was about 8-fold higher (163.3 ± 25.9 pmol/10^6 cells; $P < 0.01$). All results were checked for correlations with age of patients and controls without any significant result.

DISCUSSION

In agreement with previous investigations [18–20] this study shows that the number of high affinity β_2-adrenergic binding sites is decreased in human low grade lymphoma cells as compared with normal lymphocytes (Figs. 1 and 2). The 3-fold lower density of β_2-adrenoceptors in malignant B-cells can neither be explained by age differences [41] nor by the elevated B-: T-cell ratio in lymphoma patients as compared with normal donors, because normal B-cells bear more β_2-adrenoceptors than T-cells [20, 42, 43]. The responsiveness of AC to isoproterenol was only slightly decreased in MNL of lymphoma patients (Fig. 3). The function of the stimulating G protein, G_s, seemed to be conserved in the malignant B-cells as demonstrated by the experiments with cholera toxin (Fig. 3). These results correspond well to similar experiments on CLL lymphocytes using NaF, another stimulator of G_s [14, 18]. Finally, the most relevant finding of this study was that the AC response to forskolin was considerably impaired in the malignant B-cells (Fig. 3).

The results should be interpreted with caution because of the lack of investigations on the effect of forskolin in subpopulations of normal lymphocytes. However, the responsiveness of AC to a variety of compounds (isoproterenol, NaF, GTP and prostaglandin E_1) was shown to be *higher* in B-lymphocytes than in T-lymphocytes [20]. Therefore, it does not seem probable that the decreased AC responsiveness to forskolin in malignant B-cells may be explained by the only difference of B- and T-cells. With these limitations in mind, it can be concluded from our experiments that the β_2-adrenergic signal transduction in malignant human B-cells seems to be impaired, above all at the AC level. Additionally, the density of β_2-adrenoceptors is decreased. Both phenomena may account for decreased intracellular cAMP levels in these cells [17, 20], thereby contributing to their malignant transformation [13, 17, 22–28].

In this context, Inhorn *et al.* [28] demonstrated that the restoration of the AC responsiveness to forskolin, cholera toxin and prostaglandin E_1 by butyrate was responsible for the antiproliferative effects of these compounds in a murine myeloid cell line. The same authors provided evidence that an imbalance between the inhibitory G protein, G_i, and the stimulatory G protein, G_s, might be responsible for the decreased AC activity of this cell line. Alterations of this balance and/or functional changes of G_i would also help to explain the impairment of the AC responsiveness to forskolin observed in this study in lymphoma cells. The AC might be permanently inhibited by an absolute or relative increase of the activity of G_i [44]. Other explanations include the permanent inhibition of AC by protein

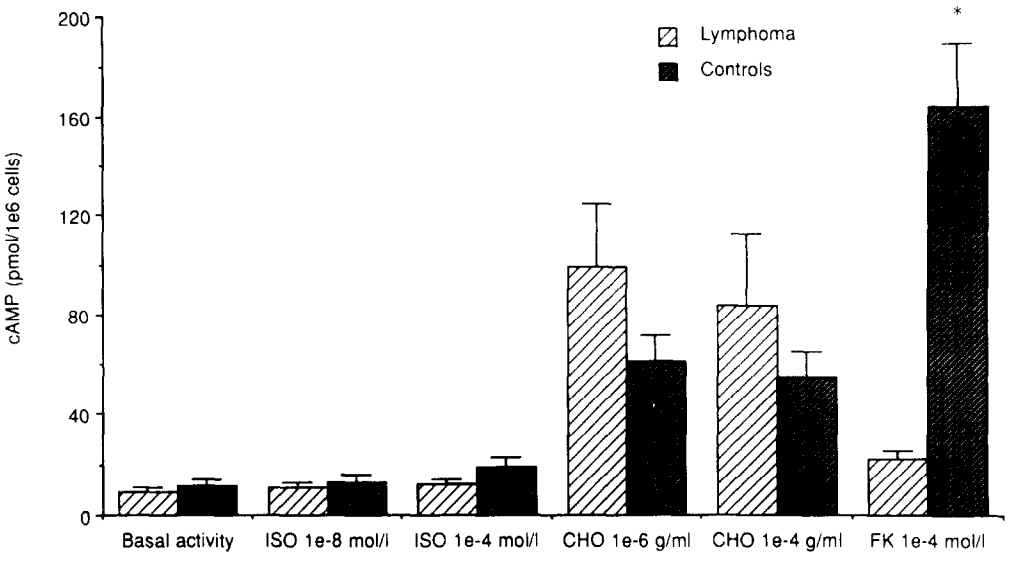

Fig. 3. Stimulation of adenylate cyclase of human lymphoma cells (N = 15) and normal lymphocytes (N = 7) by different compounds (ISO = isoproterenol, CHO = cholera toxin, FK = forksolin). For isoproterenol and cholera toxin, only two representative experiments are shown, because experiments with different concentrations or incubation periods gave similar results. * Difference from lymphoma: $P < 0.01$.

kinase C known to be stimulated by different cytokines and (tumor) growth factors [45], mutations of AC at its catalytic or regulatory sites, a reduction of AC synthesis, or an increase of AC metabolism.

However, increased inhibitory effects on AC, or changes of the catalytic subunit of AC do not explain by themselves that the stimulation of AC by isoproterenol and cholera toxin, though taking place upstream in the signal transduction pathway, seems to be less impaired than the direct AC stimulation with forskolin. One could expect that an impairment of AC should affect the signal transduction pathway to a similar extent at all levels upstream. Our experiments, however, showed no evidence that the AC impairment, as reflected by a significantly reduced responsiveness to forksolin, is necessarily paralleled by a similar decrease of its response to isoproterenol and cholera toxin in lymphoma cells. Therefore, a compensatory mechanism upstream of AC might balance the AC impairment to some extent. In our experiments, the β_2-adrenoceptor number was decreased, and the receptor affinity was not changed in lymphoma cells. Therefore, an increase of the receptor density or affinity does not seem to compensate the AC impairment. The slightly, but not significantly increased cAMP accumulation in lymphoma cells in response to cholera toxin (Fig. 3) rather supports the hypothesis that the induction of more functional G_s molecules could contribute to this compensation. However, this compensation seems to disappear with disease progression and increasing tumor cell proliferation, since the ability of cholera toxin to stimulate lymphocyte AC was found to decrease significantly with disease progression in CLL and IC patients (Hallek *et al.*, unpublished data).

The most satisfactory explanation derives from the comparison with cell line mutants defective in AC related signal transduction. Those cell lines, particularly S49 lymphoma mutants, proved to be powerful tools for studying the interaction between receptors, G proteins and AC [46–48]. Forksolin-resistant mutants of Y1 adrenocortical tumor cells [49] share some common features with our findings in lymphoma cells, and therefore merit close comparison. These mutants had a decreased responsiveness of AC to forskolin, and, though to a lesser extent, to ACTH (via the receptor) and to Gpp(NH)p, a non-hydrolysable GTP analogue. On the contrary, AC was normally stimulated by NaF (via G_s). Since the cholera toxin-mediated incorporation of [^{32}P]NAD to the α subunit of G_s (α_s) was reduced by 70%, a defect of α_s was suggested. α_s is the regulatory subunit of the G_s heterotrimer that stimulates the catalytic unit of AC [44]. It seems to be required for the full stimulation of AC by forskolin, although forskolin is able to activate AC in the absence of functional active G_s to some extent [47]. The observations on Y1 mutants show similarities to the impairment of β_2-adrenergic signal transduction reported in this study, where the cAMP accumulation was particularly decreased in response to forskolin, and to a lesser extent to isoproterenol (via the receptor) and cholera toxin (via G_s) (Fig. 3). They provide a model in which the functionally active subunit of G_s, α_s, rather than the catalytic unit of AC is altered. These alterations might account for the impairment of the β-adrenergic signal transduction pathway in human B-cell lymphoma.

These findings await further investigations involving the use of monoclonal antibodies against AC

and subunits of G proteins for quantitation by Western blotting, the study of different transmembrane signal pathways inhibiting AC, and the molecular analysis of the subunits of G_s and AC in human lymphoma cells. Further studies of the β_2-adrenergic signal transduction in lymphoma cells, with emphasis on the effects of forskolin, are under way. They may improve our understanding of the pathogenesis of lymphoma and help to develop new therapeutic strategies based on modifying the AC activity in lymphoma cells.

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