infusion for 96 h, toxicity is again very high, LD-50 being more than 50 times lower than after a single injection. Blood and tissue levels can be assumed to run a similar course after epicutaneous administration and during continuous infusion of small doses. This similarity suggests that high toxicity is related to a pharmacokinetic feature, namely long persistence of drug in the blood and/or tissues. This in turn could indicate that some in vivo metabolic process of low capacity transforms ovalicin into a highly toxic compound, and that this process is more active in guinea pigs than in the other animals tested which are less susceptible to the drug. Higher drug doses given p.o., i.v. or i.p. may be eliminated before the slow metabolism has toxified enough of the compound to produce lethality. Apparently, the toxicity of the metabolite is cumulative. A similar situation was observed with the cyclic peptide chlamydocin, which also contains an epoxy group; the LD-50 of this cytostatic compound is 23 times higher when it is injected i.v. as a bolus than when it is infused i.v. over 96 h[11]. Unfortunately, no pharmacokinetic data are available for ovalicin.

The symptoms elicited by cutaneous ovalicin administration suggest that toxicity is mainly due to an effect on the central nervous system. Whether lethality is also brought about by a central action is not clear. Since tranquilizing the drug-treated animals with a neuroleptic (thioridazine) or administration of fluid did not reduce lethality (results not shown), neither the excitation nor the adipsia provoked by ovalicin seems to be the cause of death.

The question arises, where metabolic toxification of ovalicin takes place. Is it in the liver? The comparatively high therapeutic index for the immunosuppressive activity of ovalicin suggests that the lymphatic system may be a tissue where toxification takes place. This would be compatible with the high in vitro toxicity of the compound for lymphocytes. P-815 mastocytoma cells, for which in contrast to other cell lines ovalicin is toxic too, may also convert the drug into a toxic metabolite. Is there perhaps even some toxification in parts of the central nervous system, eliciting thus the ovalicin syndrome? Such questions cannot be answered yet, but studies to elucidate these problems may lead not only to a better understanding of the effects of ovalicin but also to new ways to arrive at lymphocyte-specific, immunosuppressive compounds.

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Effect of high doses of somatostatin on adenylate cyclase activity in peripheral mononuclear leukocytes from normal subjects and from acute leukemia patients

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Summary. In normal lymphocytes somatostatin non-competitively inhibited basal (ID_{50} 5×10^{-4} M) and isoproterenol- and forskolin-stimulated adenylate cyclase activity (Ac). In acute leukemia blasts, non-responsive to isoproterenol, forskolin, which activates the catalytic subunit, stimulated and somatostatin inhibited Ac, thus indicating the leukemic enzyme, though defective, retains the inhibitory pathway and catalyst function.
Key words. Adenylate cyclase; somatostatin; forskolin; isoproterenol; lymphocytes; leukemic cells.

Somatostatin, a peptide originally isolated from the hypothalamus, as GH release inhibiting factor, has subsequently been located in many different tissues where it influences a wide variety of cellular processes[1]. Receptors for somatostatin have also been found in human mononuclear leukocytes[2] and evidence is accumulating that this peptide may modulate some lymphocyte functions. In vitro experiments have demonstrated that somatostatin at a concentration of 10^{-7} M stimulates lymphocyte proliferation and abolishes the antiproliferative effect of rat hypothalamic extracts[3], whereas at lower concentrations it clearly inhibits both spontaneous and mitogen-stimulated lymphocyte proliferation, as well as immunoglobulin synthesis induced by concanavalin A[3-6]. The mechanisms by which somatostatin affects lymphocyte functions are still unknown. In many tissues, however, the effects of the peptide have been related to its ability to inhibit cyclic AMP (cAMP) production[7-11]. It seemed therefore to be interesting to investigate the possible influence of different doses of somatostatin on adenylate cyclase activity a) in peripheral mononuclear leukocytes from normal subjects, and b) in human leukemic leukocytes, which are known to have defective adenylate cyclase unresponsive to various agents[12-16].

This paper presents evidence that in both normal and leukemic leukocytes somatostatin does not influence adenylate cyclase activity at physiological concentrations, nor at the pharmacological concentrations usually employed, but at higher doses of the peptide enzyme activity is inhibited.

Materials and methods. Eight healthy subjects and 9 untreated acute leukemia (3 lymphoblastic, ALL; 6 non-lymphoblastic, ANLL) patients with a high leukocyte count volunteered for this study. Peripheral blood was drawn with heparin as anticoagulant. As previously described[15], normal mononuclear leukocytes were separated by centrifuging the blood on Ficoll-Hypaque density gradients, whereas leukemic cells were isolated by spontaneous sedimentation at

Effect of somatostatin on basal and stimulated adenylate cyclase activity in whole extracts of peripheral mononuclear leukocytes from normal subjects and acute leukemia patients. Data are expressed as mean ± SE

	Somatostatin 5×10^{-4} M	Adenylate cyclase activity (pmoles cAMP/10 min/10^6 cells) Control	Isoproterenol 10^{-5} M	Forskolin 10^{-5} M
Normal lymphocytes	−	18.5 ± 2.14	25.9 ± 2.51[a]	100.0 ± 14.35[a]
(8 cases)	+	9.0 ± 1.15[b]	13.0 ± 1.24[a, b]	55.3 ± 11.84[a, b]
ALL leukocytes	−	3.9 ± 0.61	4.3 ± 0.85	32.2 ± 5.13
(3 cases)	+	1.8 ± 0.34	2.4 ± 0.50	21.6 ± 4.19
ANLL leukocytes	−	6.4 ± 2.16	6.8 ± 2.10	31.4 ± 8.79[a]
(6 cases)	+	3.3 ± 1.09[c]	3.9 ± 1.12[c]	19.7 ± 4.75[a, c]

[a] $p < 0.01$ vs control (Student's t-test); [b] $p < 0.01$, [c] $p < 0.05$ vs without somatostatin (two-way ANOVA).

room temperature. Cells were washed in PBS and centrifuged many times at 160 × g to remove platelets. When necessary red cells were lysed with 0.83% NH_4Cl. The preparations of normal mononuclear leukocytes (referred to in the text as lymphocytes) contained 80–90% lymphocytes, the remaining cells being monocytes and granulocytes. The preparations of ALL and ANLL cells contained 85–100% blasts. After isolation the cells, resuspended in 10 mM Tris acetate buffer (pH 7.6) and 1 mM DTT, at concentrations ranging from 25 to 50×10^6 cells/ml, were frozen-thawed once and sonicated for 10 s at 100 W. Adenylate cyclase activity was assayed in whole extracts using Salomon's method [17]. The 50-µl assay contained (final concentrations) 25 mM Tris acetate (pH 7.6), 5 mM Mg acetate, 5 mM creatinine phosphate, 2.5 U creatinine phosphokinase, 1 mM DTT, 0.01 mM GTP, 0.05 mM cAMP, 0.5 mM (α-^{32}P) ATP ($2 - 4 \times 10^6$ cpm), leukocyte sonicate (5–10 µg protein), 5 µg BSA, and, when appropriate, (−)isoproterenol ($10^{-6} - 10^{-3}$ M), forskolin ($10^{-7} - 10^{-4}$ M) and somatostatin ($10^{-14} - 10^{-3}$ M). Incubation was at 30 °C for 10 min, and was stopped by the addition of 100 µl of a solution containing 2% sodium lauryl sulfate, 45 mM ATP and 1.3 mM cAMP at pH 7.5. The (^{32}P)cAMP formed was isolated by sequential chromatography on AG50W-X4 and alumina. (^{3}H)cAMP (10,000 cpm) added before chromatography was used to monitor cAMP recoveries. All the assays were run in triplicate. Protein content was determined by Lowry's method [18]. Statistical significance of the data was evaluated by two-way ANOVA and Student's t-test.

Results. Under basal conditions adenylate cyclase activity was 2–4 times higher in normal lymphocytes than in ALL lymphoblasts, whereas there was no difference in enzyme activity between ALL and ANLL cells (table). When data were expressed as pmoles of cAMP/mg protein/min the mean values ± SE were respectively 10.4 ± 2.48 for ALL cells, 10.9 ± 4.06 for ANLL cells and 27.9 ± 4.56 for normal lymphocytes. Furthermore, both isoproterenol and forskolin stimulated adenylate cyclase activity in normal lymphocytes, but in leukemic blasts isoproterenol was generally ineffective, whereas forskolin at a concentration of 10^{-5} M retained its ability to cause a 3–8-fold increase in enzyme activity. In both normal and leukemic leukocytes somatostatin at concentrations ranging from 10^{-14} to 10^{-5} M did not influence basal adenylate cyclase activity, but at higher concentrations the peptide decreased cAMP production in a dose-dependent manner (fig. 1). When the effect of somatostatin was studied at different ATP concentrations double reciprocal plots like those of non-competitive inhibitors were found (data not shown). Fifty percent inhibition of enzyme activity occurred at a concentration of about 5×10^{-4} M. Somatostatin also inhibited adenylate cyclase stimulation induced by isoproterenol and forskolin in normal lymphocytes and by forskolin in leukemic cells (table and fig. 2). The dose-response curves in normal lymphocytes (fig. 2) indicate that the somatostatin effect was dose-dependent and that the concentration of isoproterenol (10^{-5} M) and of forskolin (4×10^{-6} M) inducing respectively maximal and half maximal stimulation of adenylate cyclase activity was apparently not influenced by the peptide, thus confirming a non-competitive mechanism of inhibition.

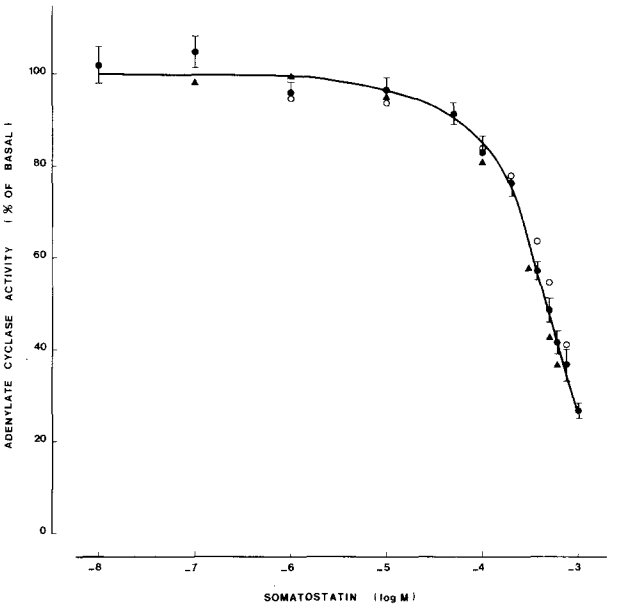

Figure 1. Effect of somatostatin on adenylate cyclase activity in lymphocytes from four normal subjects (●). Data are expressed as mean ± SE. Data relative to the blasts of one ALL (○) and one ANLL (▲) patient are also shown.

Discussion. The finding that in normal mononuclear leukocytes somatostatin inhibited both basal and stimulated adenylate cyclase activity only at high, unphysiological, concentrations suggests that the modulating influences of the peptide on lymphocyte functions [3–6] cannot be ascribed to a cAMP level decrease, but must be mediated by different mechanisms. These could include actions at steps beyond cAMP formation, influences on cyclic GMP metabolism, or changes in plasma membrane permeability to Ca^{++} and K^+, as has been postulated for various cell systems [8, 19, 20].

In line with the hypothesis that cAMP plays a role in the control of proliferative processes, alterations in the adenylate cyclase activity have previously been reported in human leukemic leukocytes, though their role in leukemogenesis is still unclear [12–16]. Further pharmacological studies could be interesting, based on the finding that the various components of the adenylate cyclase complex (membrane receptors, catalytic subunit, and transducing system) can be functionally independent and are influenced by different substances [21]. However, control validity constitutes a major problem in the

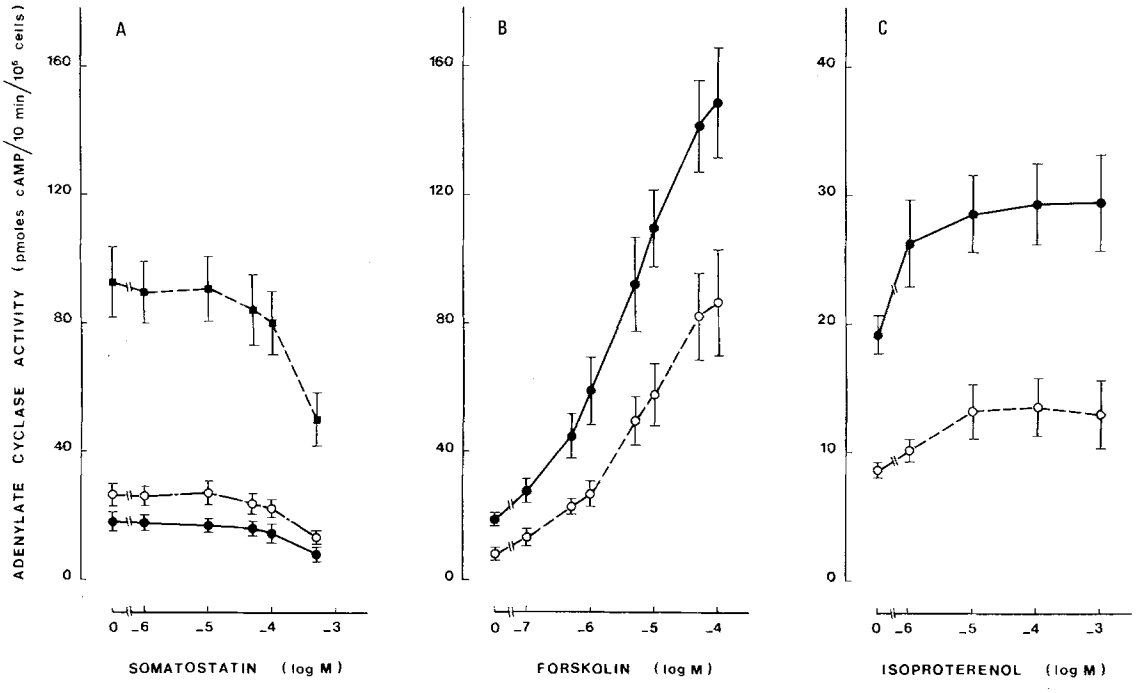

Figure 2. Adenylate cyclase dose response curves to somatostatin, forskolin and isoproterenol in lymphocytes from three normal subjects. *A* Effects of somatostatin under basal conditions (●) and in presence of 10^{-5} M isoproterenol (○) and of 10^{-5} M forskolin (■). *B* Effects of forskolin under basal conditions (●) and in presence of 5×10^{-4} M somatostatin (○). *C* Effects of isoproterenol under basal conditions (●) and in presence of 5×10^{-4} M somatostatin (○). Data are expressed as mean ± SE.

evaluation of cAMP metabolism in human acute leukemia blasts since at present normal human lymphoblasts and myeloblasts are not available for study[15]. With this limitation in mind, our results confirm that under basal conditions a low adenylate cyclase activity characterizes both ALL and ANLL cells which also show a markedly decreased responsiveness to isoproterenol, probably due to a reduction in adrenergic receptors[13].

This alteration at the plasma membrane surface may not, however, account for the reduced basal adenylate cyclase activity, which could reflect a decreased number of enzyme sites[14] or an impaired catalyst function. This latter possibility seems however improbable since we found that forskolin, a diterpene that stimulated adenylate cyclase by directly interacting with the catalytic subunit of the enzyme[22], induced a 3–8-fold increase in enzyme activity in both normal and leukemic leukocytes. It is at present unknown whether human leukemic blasts retain membrane receptors for somatostatin, but in our experiments leukemic cells showed adenylate cyclase responses to the peptide similar to those of normal leukocytes. However, the high concentrations of somatostatin necessary to inhibit leukocyte adenylate cyclase activity suggest that the mechanism involved might not be receptor-mediated, whereas a possible influence of the peptide on the transducing system or the catalytic subunit cannot be excluded.

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