

## Neuroimmunomodulation of Natural Killer (NK) Cells by Ergot Alkaloid Derivatives

A. FIŠEROVÁ<sup>1</sup>, H. KOVÁŘŮ<sup>4</sup>, Z. HAJDUOVÁ<sup>5</sup>, V. MAREŠ<sup>3</sup>, M. STAREC<sup>6</sup>, V. KŘEN<sup>2</sup>, M. FLIEGER<sup>2</sup>, M. POSPÍŠIL<sup>1</sup>

<sup>1</sup>Department of Immunology, <sup>2</sup>Biogenesis of Natural Products, Institute of Microbiology, <sup>3</sup>Department of Neuroscience, Institute of Physiology, Academy of Sciences of the Czech Republic, <sup>4</sup>Research Psychiatric Unit, First Faculty of Medicine, <sup>5</sup>Faculty of Natural Sciences, and <sup>6</sup>Department of Pharmacology, Third Faculty of Medicine, Charles University, Prague, Czech Republic

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### Summary

Ergot alkaloids (EAs), products of *Claviceps spp.*, are widely used in various fields of clinical medicine (neurology, psychiatry, endocrinology). In the present work we studied the neuroimmunomodulative effect of EAs on activation of NK cells and their signalling pathways. Furthermore, the killing capability of rat NK cells *in vitro* was examined in the presence of glycosidic derivatives of elymoclavine, agroclavine, and liposome-encapsulated EAs. The engagement of appropriate NK cell membrane receptors by EAs cause an indirect enhancement of adenylyl cyclase system through inhibition of G-protein  $\alpha_{1,2}$ -subunit (up to 50 % of control values). All of the tested EAs enhanced the rat NK cell-mediated cytotoxic activity *in vitro*, particularly against target cells of astrocyte origin (C-6 glioma). The present results argue for a possible EA immunomodulatory role of cell-mediated immunity in tumour regression processes.

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### Key words

Natural killer cells – Ergot alkaloids – Neuroimmunomodulation

### Introduction

A wide range of biological effects of EAs in neural and endocrine tissues could be explained on the basis of the structural similarities with important mediators of neurotransmission, such as receptor for biogenic amines (Muller-Schweinitzer and Weidmann 1978, Hadden 1987). The communication between the neuroendocrine and immune systems is mediated by neurotransmitters and corresponding receptors on the cells of both neural and immune origin. From the functional point of view, these effects are mediated by adrenergic (Aarons *et al.* 1983, Hellstrand and Hermodsson 1989), dopamine (Deleplanque *et al.*

1994) or serotonin receptors (Hellstrand and Hermodsson 1990). The lymphoid cells possess membrane receptors for a variety of hormones that affect DNA synthesis and proliferation *via* the ubiquitous cAMP (cGMP) systems (Chelmicka-Schorr and Arnason 1990). Generally, the process of signal transduction such as hormonal or neurotransmitter action often operates through cell receptors coupled to membrane adenylyl cyclase (Remaury *et al.* 1993). The cyclic nucleotide functioning as a transmitter of the hormonal message to the genome, thereby resulting in a change in the physiological status of the cell in question (Davis *et al.* 1992).

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Results were preliminary presented at "CNS – Advances in Research of Normal and Neoplastic Cells" which was held in Brno (April 25, 1996) as the satellite minisymposium of the 42nd International Congress of the European Tissue Culture Society (Fišerová *et al.* 1996).

Corresponding receptors to neurotransmitters have been described in mature and developing lymphocytes (Singh *et al.* 1987). Neurotransmitters may, hitherto, modulate immune functions, e.g. T cell differentiation, NK cell function (Hellstrand *et al.* 1990), T and B cell response to lectins, macrophage functions, etc. (Singh 1985). For this reason we have focused on the determination of direct effects of EAs on some immune functions.

## Materials and Methods

### Materials

Elymoclavine (E) and agroclavine (A) used in this study were kindly donated by Galena Pharmaceuticals Ltd. (Opava, Czech Republic). New glycosides derived from elymoclavine were synthesized by chemoenzymatic methods.  $\beta$ -Glucosides were synthesized either by chemical method or by transglucosylation (glycosidase from *Aspergillus oryzae*). Preparation of  $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ O)-elymoclavine (E-Glc),  $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ O)-elymoclavine (E-Gal), 2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ O) elymoclavine (E-GlcNAc) and 2-acetamido - 2-deoxy -  $\beta$ -D-galactopyranosyl- (1 $\rightarrow$ O)-elymoclavine (E-GalNAc) were prepared according to Křen *et al.* (1994). Phytohemagglutinin (PHA) and recombinant human interleukin-2 (rIL-2) were purchased from Sigma (St. Louis, USA). Recombinant human interleukin-12 (rIL-12) and neutralizing antibodies against IL-2 and IFN- $\gamma$  were kindly provided by Dr. G. Trinchieri (Wistar Inst., Philadelphia, USA).

Sterically stabilized long circulating liposomes with encapsulated drugs were prepared according to the procedure of Vaage *et al.* (1992). Composition in molar ratio was following: 56.1 : 38.2 : 5.5 : 0.2 – hydrogenated soy phosphatidylcholine : cholesterol : PEG-DSPE (polyethylene glycol-derivatized distearoyl phosphatidylethanolamine) : tocopherol. Liposomes were conjugated with oligosaccharides (natural ligands of NKR-P1 lectin-like receptors) and/or agroclavine.

Long-term cultures and all *in vitro* experiments were performed in an RPMI-1640 medium supplemented with L-glutamine (2 mM), antibiotics (Penicillin 100 U/ml, Streptomycin sulfate 100  $\mu$ g/ml, Amphotericin B 25  $\mu$ g/ml) and with 10 % foetal calf serum (FCS). Incubation was carried out at 37 °C in a humidified atmosphere containing 5 % CO<sub>2</sub>.

### Cell preparations

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors on Ficoll-Hypaque (Sigma, St. Louis, USA) density gradient (1.078). The cells were counted and resuspended in a complete culture medium. An enriched human NK cells population was obtained by cocultivation of PBMC with  $\gamma$ -irradiated RPMI8866

(human B lymphoma cell line) according to the procedure of Perussia *et al.* (1987).

The *in vitro* cytotoxicity experiments were performed with RNK16 rat NK cell line as effector cells. The YAC1 (mouse myeloma), P815 (mouse mastocytoma) and C-6 (rat glioma) cell lines served as target cells for the cytotoxicity assays.

### Assays used for determination of G-protein $\alpha$ -subunit expression

The analysis of EA-mediated cell signalling pathways in G-protein  $\alpha$ -subunit expression was performed on the RNK16 cell line and human NK cells as described above. The level of  $\alpha$ -subunit expression in cholate membrane extracts were estimated by ELISA microplate competitive inhibition immunoassay. A modified method of Ransnäs and Insel (1989) on the Maxisorp microtitration plates (NUNC) by noncovalent peptide binding was used (Kovářů *et al.* 1995). The polyclonal rabbit antisera against C-terminal decapeptides of  $\alpha$ i1,2,  $\alpha$ s and  $\alpha$ q/11 subunits (Goldsmith *et al.* 1987) prepared in our laboratory were used. No cross-reactivity was found between antisera in ELISA tests.

### Cell proliferation assay

Proliferation induced by EAs and other biologicals (PHA, IL-2, IL-12) on human PBMC and NK cell populations was measured after 3 days in culture. All inducers and antibodies were added to a final volume of 250  $\mu$ l in flat-bottomed tissue culture microtiter plates. [<sup>3</sup>H]-methyl thymidine ([<sup>3</sup>H]-TdR, 2 mCi/mM of specific activity, UVVVR, Prague) was added (1  $\mu$ Ci/well) for the last 4 hours of incubation. Cells from triplicates were harvested and [<sup>3</sup>H]-TdR incorporation was measured as described elsewhere (Fišerová *et al.* 1995a).

### Cytotoxicity assay

Effector cells at concentrations  $2 \times 10^5$ /well were incubated in 96-well round-bottomed tissue culture plates with  $10^4$ /well of <sup>51</sup>Cr-labelled (60 min) target cells in a final volume of 250  $\mu$ l. The cytotoxic activity against NK-sensitive YAC1 as well as against NK-resistant P815 and C-6 target cells was measured by standard 4 or 20 hours <sup>51</sup>Cr-release assay as reported previously (Fišerová *et al.* 1995a,b).

## Results

### NK-cell signal transduction mediated by EAs

Numerous neurotransmitters, peptide hormones and neuromodulators elicit changes in cellular metabolism by interaction with cell membrane receptors that are coupled to intracellular effector enzymes by GTP-binding proteins (G-proteins).

Another point relevant to the question of receptor-ligand interactions is that many hormones, neurotransmitters and also ergot alkaloids share similar chemical features (Eich and Pertz 1994).

The G-protein  $\alpha_{i1,2}$ -,  $\alpha_s$ - and  $\alpha_q/11$ -subunits were determined in NK cell membrane preparations of the rat RNK-16 cell line and human NK-enriched populations. Agroclavine was added in  $10^{-6}$  M concentration to the cell suspensions ( $5 \times 10^7$  cells/sample/25 ml) for 18 hours prior to the isolation of membranes. Control cultures were maintained under the same conditions. Table 1 indicates only marginal inhibition of  $\alpha_{i1,2}$  and  $\alpha_q/11$  subunit expression by agroclavine in the rat cell system, but up to 50% inhibition in human NK cells. However,  $\alpha_s$ -subunit expression was not influenced in either rat or human systems under these experimental conditions.

**Table 1.** Agroclavine effect on expression of G-protein  $\alpha$ -subunits in rat and human NK cells

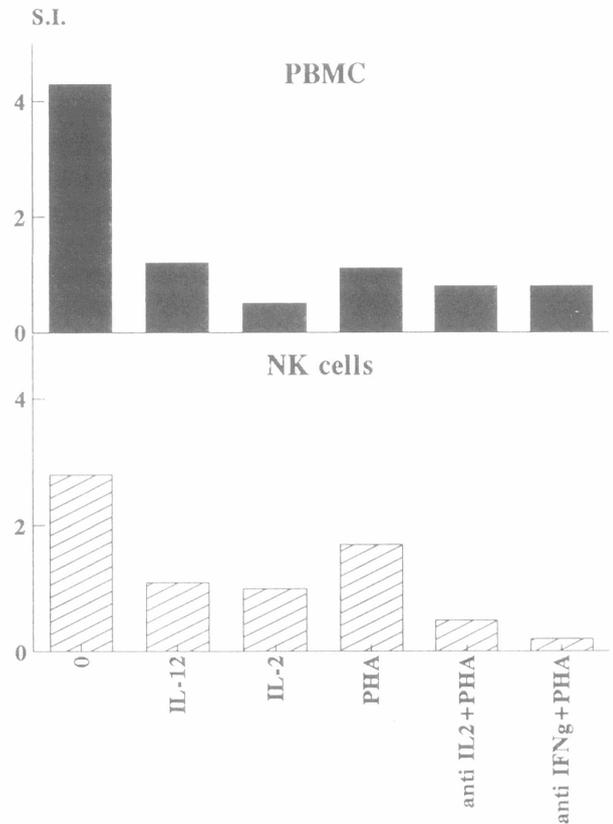
Subunit	Rat RNK16 cells	Human NK cells
G $\alpha_q/11$	71.4 $\pm$ 8.25 %	49.2 $\pm$ 6.8 %
G $\alpha_s$	98.8 $\pm$ 10.4 %	106.2 $\pm$ 11.6%
G $\alpha_{i1,2}$	86.3 $\pm$ 9.5 %	56.8 $\pm$ 6.9 %

Agroclavine was added in  $1 \mu\text{M}$  concentration to the NK cells for 18 hours. Microsomal membranes were isolated ( $4-5 \times 10^7$  vesicles/sample) and treated with 1% cholate. Cholate extracts ( $5-20 \mu\text{g}/\text{well}$ ) were used for ELISA. The results are expressed in percentage of control values  $\pm$  S.E.M.

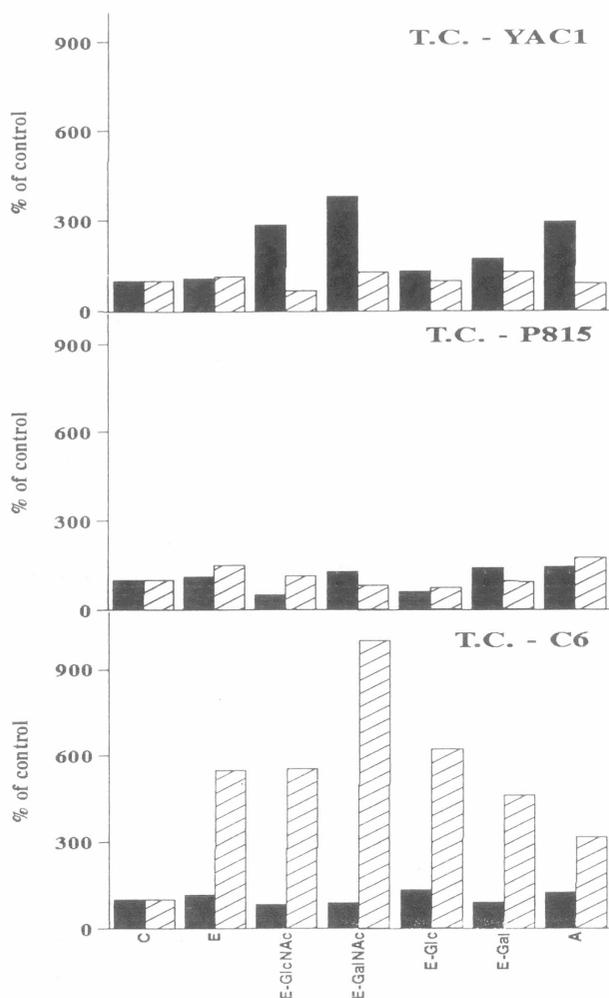
#### Effect of agroclavine on the proliferation of human PBMC and NK cells

For a better understanding of how EAs influence the proliferation of lymphocytes, we determined whether agroclavine alone, or in combination with lymphokines (IL-2, IL-12) and mitogens (PHA), affects the mitotic activity of purified NK cells. Concomitantly, we analyzed the proliferation of unseparated PBMC cultured for 3 days. Agroclavine added to the culture had a stimulating effect on both NK and PBMC and exerted an additive proliferative effect on the population of NK cells induced by PHA. To investigate the possibility that the synergistic effect of agroclavine with PHA-induced proliferation of NK cells is due to induction of cytokines such as IL-2 or IFN $\gamma$ , we tested the ability of neutralizing antibodies against both cytokines to affect the proliferation of the cells (Fig. 1). Both anti-IL-2 and anti-IFN $\gamma$  antibodies blocked markedly the proliferation of NK cells induced by PHA in the presence of agroclavine. Similarly,

IL-2-induced proliferation of PBMC was inhibited by agroclavine but the mitotic activity under PHA remained without changes. From these and previously published results we can assume the sensitivity of NK cells to agroclavine (Fišerová *et al.* 1995a).



**Fig. 1.** The effect of agroclavine on the lymphokine- and mitogen-induced proliferation of PBMC and NK cells. The role of anti-IL-2, anti-IFN $\gamma$  antibodies on PHA-induced proliferation. PBMC and purified NK cells were cultured with PHA (1%), rIL-2 (100 U/ml) and rIL-12 (1 ng/ml) in the presence or absence of agroclavine (1 nM). Anti-IFN $\gamma$  monoclonal antibody (B133.3 ascites  $10^{-3}$  dilution, neutralizing  $10^6$  U IFN $\gamma$ /ml) or goat anti-IL-2 polyclonal antibody (serum,  $10^{-2}$  dilution, neutralizing  $10^3$  U IL-2/ml) were added to the indicated cultures. Cell proliferation was measured on the 3rd day of culture as [ $^3\text{H}$ ]-TdR incorporation, reported in stimulation index (S.I. = experimental cpm/control cpm). The control values and stimulatory biological values are given as S.I. = 1.00 and the proliferative changes in the presence of agroclavine are subtracted from these values. S.I. of stimulated cultures were following: PBMC: PHA = 97.5, IL-2 = 142.0, IL-12 = 3.5, anti-IL-2 + PHA = 108.0, anti-IFN $\gamma$  + PHA = 176.7; NK cells: PHA = 9.5, IL-2 = 229.3, IL-12 = 40.5, anti-IL-2 + PHA = 3.4, anti-IFN $\gamma$  + PHA = 22.0. [ $^3\text{H}$ ]-TdR incorporation in control unstimulated cultures (medium) was 850 cpm. Results are expressed as means of 3 experiments performed.



**Fig. 2.** The effect of EA-glycosides on cytotoxic activity of rat NK cells. RNK-16 NK cell line as effector cells (E.C.) and YAC1, P815 (haematopoietic origin) or C-6 glioma (astrocyte origin) as target (T.C.) cells were used in  $^{51}\text{Cr}$ -release assay. Two time periods 0–4 hours (full columns) and 4–20 hours (hatched columns) were measured. The E.C./T.C. ratio was 20:1. Results are expressed in % of control values. Cytotoxicity control values (medium) for different target cells were following: YAC1 = 18.4, P815 = 1.5, C-6 = 2.7. The data are representative of six experiments performed with similar results. For abbreviations see Methods

#### NK cell-mediated cytotoxicity in the presence of EAs *in vitro*

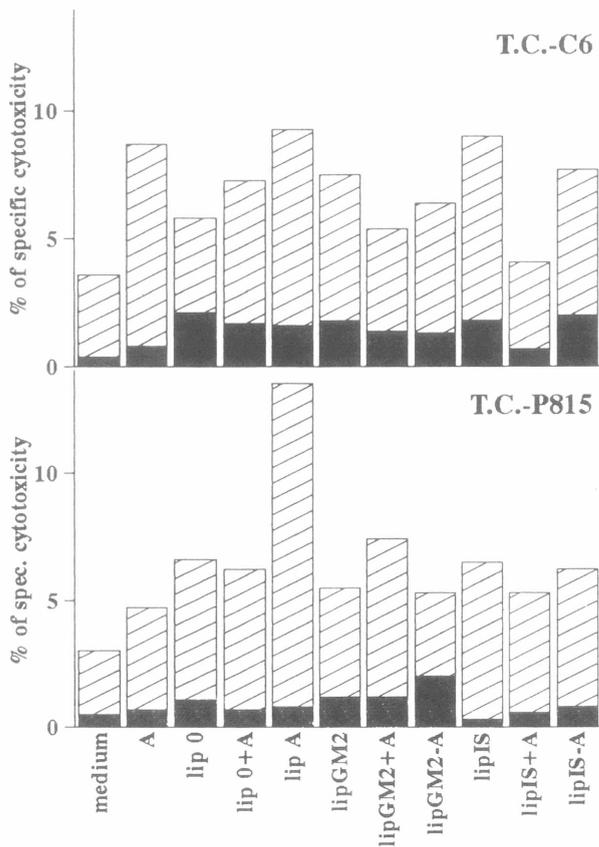
NK cells have a potential to lyse a wide array of tumour cells *in vitro* and *in vivo* (Trinchieri 1989). The immunostimulatory role of various elymoclavine glycosides in comparison with aglycon and another natural clavine type of ergot alkaloid – agroclavine was

tested on the cytotoxic activity of NK cells. Previously, we reported that EAs potentiate the NK cell cytotoxicity induced by oligosaccharide ligands of C-type lectin receptors. However, in comparison with aglycon, EA-glycosides increased enhancement of the cytotoxic activity of human PBMC against both NK-sensitive and NK-resistant target cells. This was dependent on both alkaloid and saccharide part of the molecule (Křen *et al.* 1996).

To investigate the mechanism of possible involvement of neurotransmitter receptors in the cytotoxic reaction, we used target cells of astrocyte origin (C-6 glioma). The lymphoma (YAC1) and mastocytoma (P815) target cell lines were simultaneously tested in 4 and 20 hours  $^{51}\text{Cr}$ -release assay. Figure 2 shows the highest enhancement of NK cell-mediated cytotoxic activity in short-term (0–4 hours) assay against YAC1 target cells by GalNAc and GlcNAc derivatives of elymoclavine and by agroclavine. The P815 and C-6 target cells remained resistant. However, in 20 hours lasting assay the highest killing capability of NK cells in the presence of EAs was observed against target cells of astrocyte origin (C-6). This effect was elicited especially by N-acetylgalactosamine derivative of elymoclavine.

#### NK cell-mediated cytotoxicity influenced by liposome-encapsulated agroclavine and oligosaccharides *in vitro*

Following the facts that liposome-entrapped high-affinity oligosaccharide ligands of NKR-P1 exhibit stimulation of NK cell-mediated cytotoxicity (Bezouška *et al.* 1994b) and EAs ameliorate the response through neurotransmitter receptors, we utilized the combination of both substances incorporated into the liposomes. Ligands of NKR-P1 include oligosaccharide sequences for ganglio family (GM2) and heparin type oligosaccharide (Heparin IS). Rat RNK-16 cell line as effector and P815 or C-6 cell lines as target cells were used in cytotoxicity assay. Liposomes were prepared by using GM2 or IS with incorporated agroclavine (lipGM2-A, lipIS-A). They were compared with liposomes containing 0.02  $\mu\text{M}$  of either agroclavine or neoglycolipids (GM2 or IS) separately. Liposomes and/or free agroclavine (+A) were added in 50  $\mu\text{l}$  of a total volume 250  $\mu\text{l}$ . Results obtained with liposomes containing either both or single substances (Fig. 3) indicated only a weak enhancement of cytotoxicity with both substances as compared to the action of agroclavine-encapsulated liposomes. However, the killing capability of effector cells was markedly increased (3.7 times) with liposome-encapsulated agroclavine against P815 target cells (after 20 hours of incubation). The increased killing capability of NK cells against C-6 glioma target cells in the presence of agroclavine was not dependent on liposome technology (compare A and lipA in Fig. 3).



**Fig. 3.** The effect of liposomes with incorporated agroclavine in the presence or absence of oligosaccharides followed in cytotoxicity assays *in vitro*. RNK-16 NK cell line as effector cells (E.C.) and C-6 glioma or P815 as target cells (T.C.) were used in  $^{51}\text{Cr}$ -release assay. Two time periods 0–4 hours (full columns) and 4–20 hours (hatched columns) were measured. The E.C./T.C. ratio was 20:1. Results are expressed in % of specific cytotoxicity. The data are representative of three experiments performed with similar results. For abbreviations see Results.

## Discussion

Converging evidence from the field of neuroendocrinology has demonstrated that the immune system is not regulated exclusively in an autonomous fashion, but is influenced by external factors directed by the CNS. Furthermore, cells of the immune system express receptors for neuroendocrine mediators or include active transporter systems for neurotransmitters (Faraj *et al.* 1991). Ergot alkaloids in relation to their structural similarities with biogenic amines can exert an agonistic or antagonistic action on the corresponding receptors (Eich and Pertz 1994). NK cell activities are considered as a most sensitive parameter to neuroimmunomodulatory effects of EAs. In addition, ergot alkaloid glycosides are hybrid

molecules which could be bound to the receptors for biogenic amines as well as to lectin receptors (proteins with specific affinity to carbohydrates).

To test this hypothesis, the experiments described in the present paper were performed using rodent target cells of haematopoietic (YAC1, P815) as well as astrocyte (C-6) origin. The C-6 target cells express a higher number of neurotransmitter receptors and perhaps are more sensitive to the regulation by the central and peripheral nervous systems than the lymphoid target cells. From binding and inhibition studies we are familiar with rat NK cells. The most common ligands of rat NKR-P1 receptor expressed by rat effector cells are represented by GalNAc or GlcNAc monosaccharides or by naturally occurring oligosaccharides containing such terminal moieties (Bezouška *et al.* 1994a).

An interesting question is whether cell surface molecules other than those designated for antigen capture can serve in antigen recognition and delivery to the processing effector compartment (killing capability). In addition to the efficient antigen capture (carbohydrate recognition), EAs may fulfil other requirements for antigen presentation and NK cell stimulation (some EAs can solely stimulate cell-mediated cytotoxicity). Our working hypothesis that glycosylated EAs as GalNAc-elymoclavine derivative should be the more efficient compound, was confirmed by the results showing the highest stimulatory activity of NK cell-mediated killing of C-6 glioma cells *in vitro*.

Thus the crucial question arising from these experiments concerns the nature of receptors, signal transduction and mechanism(s) involved in effector-target interaction. It appears from the cytotoxicity and other experiments (binding studies – data not shown) that EAs act by another mechanism than those involved in direct binding and triggering of the effector cells. This finding is also supported by the fact that the cytotoxic effect on C-6 targets occurs after 20 hours, i.e. later than on the lymphoid targets. We know from the preliminary results that the ergot alkaloids bind to the nuclear and cytoplasmic fractions of lymphocytes and tumour cells, but the mechanism of cell activation is still unknown. Thus, ergot alkaloids can also be recognized by target cells, namely of neural origin (C-6 glioma cells), and by this way they participate in the effector-target interaction. This is in concert with the experiments provided by agroclavine-conjugated liposomes that exerted a prolonged effect on the killing of specialized targets.

Other experiments were conducted to determine whether intracellular communication acting through G-protein coupled receptors results in modulation of phospholipase C activity or adenylyl cyclase systems. The cAMP, as a "second messenger", can influence the transmission of growth signals through the *Ras* pathway. The crosstalk between cAMP and other intracellular regulatory kinases (PKA)

influence the cell growth by stimulatory or inhibitory action (Marx 1993). The treatment of NK cells by agroclavine caused an indirect enhancement of adenyl cyclase activity through inhibition of G-protein  $\alpha_{i1,2}$ -subunit expression, being higher in human NK cells ( $\alpha_{i1,2}$ - and  $\alpha_q/11$ -subunits). This may be associated with increased functional activity as an alternative mechanism involving more selective signalling pathways.

Our findings provide the biological support for the notion that EAs participate in the signal transduction of NK cells which follows the recognition

of tumour targets. However, it is unlikely that EAs solely participate in the signalling through the NK receptor(s) for target recognition. Research establishing the answers to these questions will provide a useful basis for better understanding of EA role in cytotoxicity events.

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**Reprint requests**

A. Fišerová M.D., Ph.D., Department of Immunology and Gnotobiology, Institute of Microbiology, Vídeňská 1083, 142 20 Prague 4, Czech Republic.