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CYTOTAXIN-INDUCED cAMP PEAK IN GRANULOCYTES: ITS RELATIONSHIP TO CRAWLING MOVEMENTS, CHEMOKINESIS AND CHEMOTAXIS

A. NAEF,* B. DAMERAU† and H. U. KELLER*

*Institute of Pathology, University of Berne, Freiburgstrasse 30, Berne, Switzerland, and †Max-Planck Institut für experimentelle Medizin, Abteilung Biochemische Pharmakologie, D-3400 Göttingen, West Germany

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Abstract—The relationship between the short transient intracellular increase in cAMP levels on the one hand and chemotaxis or crawling movements on the other hand was investigated using human and equine granulocytes. $C5a_{des\ arg}$, f-met-leu-phe, human serum albumin and immunoglobulin were used as stimulating agents. There was no strict correlation between the induction of crawling movements or of chemokinesis in general and the generation of the cAMP peak. But there was so far a strict parallelism between the occurrence of the chemotactic response and the cAMP peak. However, the magnitude of the peak was not representative for the extent of directional locomotion. It seems possible that cAMP is an essential step in the transduction of the chemotactic response, but there is no proof for a causal relationship as yet.

The biochemical pathways, which are involved in the transduction of a chemotactic signal have been investigated extensively. Many experiments were concerned with the putative role of cyclic nucleotides. Several studies showed that a sustained increase of cAMP levels was associated with inhibition of directional locomotion [1–5] and that exogenous dibutyryl cAMP [2, 6, 7] and cAMP [2, 5, 8] inhibited the directional locomotion. The results lead to the conclusion that although cAMP is not in the main sequence of events triggering the chemotactic response, increases in cAMP may modulate directional locomotion.

More recent studies demonstrated a short transient increase in cAMP levels following stimulation with cytotaxins [9–13]. The question as to whether cAMP might be involved in the transduction of the chemotactic signal has to be reconsidered. Cytotaxins may produce a variety of biological effects such as changes in membrane potential, chemokinesis, chemotaxis, exocytosis in presence of cytochalasin B, a metabolic burst, superoxide formation, and others. The purpose of the present paper was to investigate the relationship between the short transient cAMP increase and stimulation of crawling movements, chemotaxis and chemokinesis.

MATERIALS AND METHODS

Compounds. Human serum albumin (HSA) was obtained from Behringwerke (Marburg, West Germany), (Lot No. 455012 A). The standard gamma globulin (SGG) preparation obtained by ethanol precipitation [14] was a gift of the Swiss Red Cross Transfusion Centre (Prof. A. Hässig). The lyophilised preparation was dissolved in and dialysed against Gey's solution at 4° overnight. The synthetic peptide f-met-leu-phe (FMLP) and gly-his-gly were purchased from Sigma Chemical Co., (St. Louis, MO). The competitive antagonist of the f-met-peptides carbobenzoxyphenylalanyl-methionine (CBZ-phe-met) was obtained from Fluka AG (Buchs, Switzerland). FMLP and CBZ-phe-met were dissolved in dimethylsulfoxide (DMSO) and stored at −20° until use. DMSO alone at the final concentration used in the experiments had no effect on directional locomotion. $C5a_{des\ arg}$ was isolated from hog serum as described by Grossklaus *et al.* [15].

Cell preparation. Peripheral blood was collected from healthy human donors (Dr. Deubelbeiss, Swiss Red Cross Transfusion Service, Berne, Switzerland) and from healthy horses (generously supplied by Dr. P. Arnold, Tierspital, Berne, Switzerland). Granulocytes were separated from heparinized blood (10 units heparin/ml) by means of Ronpacon–Methocel and subsequent passage through a discontinuous Ficoll–Ronpacon gradient [16]. The cell population containing more than 96% granulocytes was washed twice and resuspended in Gey's solution containing 2% HSA for assessment of crawling movements, random and directional locomotion and cAMP assay.

Stimulation of granulocytes and cAMP assay. The cell suspension (5×10^7 granulocytes/ml) was placed in a siliconised glass tube and stirred at 37° (water bath). After addition of an appropriate volume of the 100-fold concentrated stock solution of the reagent in question, 40 μl of the suspension was mixed with 160 μl 1.4 N perchloric acid at the time indicated and immediately vortexed. After centrifugation the supernatant was neutralized by addition of 6 N K_2CO_3, centrifuged and diluted in the range from ten to thirty times with sodium acetate buffer (pH 6.2). The cAMP content was measured by radioimmunoassay (cAMP [^{125}I]RIA kit; New England Nuclear, Boston, MA) according to the protocol for acetylated samples. Extracellular and intracellular cAMP, respectively, were measured as follows: 200 μl dibutylphthalate was layered on 100 μl 1.4 N

perchloric acid. Then, an aliquot (40 μl) of the cell suspension was layered on dibutylphthalate and immediately centrifuged in an Eppendorf microcentrifuge. The separation was complete after 8 sec. The cell suspension was therefore layered on dibutylphthalate 8 sec before the time indicated.

Assessment of random and directional locomotion and chemotaxis. Migration was measured in triplicate by the two filter count method [17] using modified Boyden chambers incubated for 3 hr to measure random migration (no gradient) or 1 hr to assess directional locomotion (positive gradient) at 37°. The chemotactic properties of $C5a_{des\ arg}$ and FMLP have been demonstrated by visual orientation assays [18, 19]. We therefore extrapolate that gradients of f-met-leu-phe and $C5a_{des\ arg}$ induce directional locomotion in the filter assay as well. Visual assays showed that cells in absence of a gradient change their direction at random and there is indirect evidence that this also occurs in the filter assay. [20, for details of terminology see 21]. SGG and native HSA produced no orientation.

Assessment of shape changes of granulocytes. Granulocytes (10^6/ml) were suspended in the appropriate test solution and preincubated for 30 min in a water bath with a reciprocating shaker at 37°. They were transferred into a prewarmed (37°) Sykes–Moore chamber and their behaviour in suspension was immediately recorded using phase-contrast microscopy, a Newvicon television camera and a JVC tape recorder. Adhesion patterns visualized by reflection-contrast microscopy [22], as well as motility and cell shape (non-motile spherical or spread without polarity and motile polarized) visualized by phase-contrast microscopy of the leucocytes settled on the coverslip, were recorded 30 min later and analysed as described by Keller *et al.* [23].

RESULTS

Assessment of intra- and extracellular cAMP. Intracellular and extracellular cAMP were measured before and after stimulation of granulocytes with $C5a_{des\ arg}$. The cAMP peak appearing within 1 min after stimulation is essentially associated with the cells (data not shown). Only about 5–10% of the cAMP measured is found in the medium.

Stimulation of granulocytes with cytotaxins and non-chemotactic analogues. Intracellular cAMP levels were measured before and after exposure of human granulocytes to chemotactic concentrations of either $C5a_{des\ arg}$ (6.7×10^{-8} M) or f-met-leu-phe (5×10^{-8} M). A two to three fold increase was observed within 1 min following stimulation. CBZ-phe-met, which was not chemotactic for human neutrophils (data not shown), did not significantly increase the cAMP level (Fig. 1). It had been reported that the peptide gly-his-gly is chemotactic and has no effect on the cAMP level. Yet our experiments showed that gly-his-gly had very little if any chemokinetic effect, but it failed to induce directional locomotion (data not shown).

The response of equine neutrophils was also tested. f-met-leu-phe was not chemotactic and it produced no transient cAMP increase in these cells.

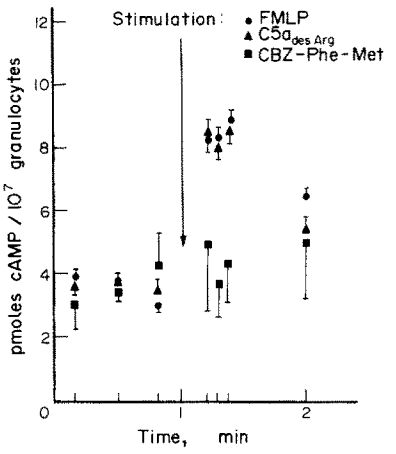

Fig. 1. Measurement of cAMP before and after stimulation of neutrophils with $C5a_{des\ arg}$, f-met-leu-phe (FMLP) and CBZ-phe-met. Human neutrophils in Gey's solution containing 2% HSA were stimulated with $C5a_{des\ arg}$ (final concentration 6.7×10^{-8} M), FMLP (final concentration 5×10^{-8} M) and CBZ-phe-met (final concentration 10^{-4} M). Data represent the mean of 16 (●), 17 (▲) and 3 (■) individual experiments. $C5a_{des\ arg}$ and FMLP did significantly increase cAMP levels ($P < 0.001$). In the presence of CBZ-phe-met the cAMP level was not significantly different ($P > 0.1$) from its respective control.

In contrast, $C5a_{des\ arg}$ was chemotactic for equine neutrophils and induced a cAMP peak (Table 1).

Quantitative relationship between transient cAMP peak and chemotactic response. The following restimulation experiments were designed to study whether the chemotactic response of neutrophils correlates quantitatively with changes in the cAMP peak. In a first series of experiments neutrophils were exposed to $C5a_{des\ arg}$ (6.7×10^{-8} M) or f-met-leu-phe (5×10^{-8} M) for 1 min, washed and restimulated with the same cytotaxin at identical concentrations. The cells showed no decrease in directional locomotion (Table 2) whereas the cAMP level after restimulation was reduced by 62 and 32%, respectively (Fig. 2). The increase in cAMP after restimulation was still statistically significant.

In order to achieve chemotactic deactivation, neutrophils were preincubated in $C5a_{des\ arg}$ at a higher concentration (3.3×10^{-7} M) at 37° for 30 min, washed and restimulated with a lower concentration of $C5a_{des\ arg}$ (6.7×10^{-8} M) 20 min later. Such neutrophils showed unimpaired random locomotion but reduced directional locomotion (Table 2). The transient cAMP peak was strongly reduced after restimulation with $C5a_{des\ arg}$ (Fig. 3).

Relationship between chemokinesis, crawling movements and transient cAMP increase. Human serum albumin as well as immunoglobulins (SGG) can produce marked chemokinetic effects in granulocytes. The HSA utilised for this study did not induce crawling movements of neutrophils in suspension (Table 3). Its chemokinetic effect was associated with decreased adhesion of the cells to the substratum (data not shown). In contrast to native HSA, immunoglobulin preparations (SGG) induced crawling movements in granulocytes (Table 3). Neither HSA nor SGG produced a peak of cAMP

Table 1. Effect of f-met-leu-phe (FMLP) and $C5a_{des\ arg}$ on cAMP level, on directional and random locomotion of equine neutrophils

Test solution	Medium for cell suspension	Incubation time of Boyden chambers (hr)	Per cent neutrophils migrated (± S.D.)	pmoles cAMP/10^7 granulocytes ± S.E.*
2% HSA–Gey's	2% HSA–Gey's	1	0.1 ± 0.07	1.5 ± 0.02
$C5a_{des\ arg}$, 3.3×10^{-8} M	2% HSA–Gey's	1	23.8 ± 3.6	7.9 ± 2.5
FMLP, 10^{-10} M	2% HSA–Gey's	1	0.03 ± 0.03	1.8 ± 0.8
10^{-9} M	2% HSA–Gey's	1	0.06 ± 0.01	1.1 ± 0.4
5×10^{-8} M	2% HSA–Gey's	1	0.1 ± 0.04	1.8 ± 0.5
10^{-7} M	2% HSA–Gey's	1	0.1 ± 0.05	2.2 ± 0.9
10^{-6} M	2% HSA–Gey's	1	0.05 ± 0.02	1.7 ± 0.5
10^{-5} M	2% HSA–Gey's	1	0.03 ± 0	2.1 ± 0.7
2% HSA–Gey's	2% HSA–Gey's	3	0.4 ± 0.2	
$C5a_{des\ arg}$, 3.3×10^{-8} M	$C5a_{des\ arg}$, 3.3×10^{-8} M	3	1.0 ± 0.7	
FMLP, 10^{-10} M	FMLP, 10^{-10} M	3	0.3 ± 0.3	
10^{-9} M	10^{-9} M	3	0.1 ± 0.08	
5×10^{-8} M	5×10^{-8} M	3	0.4 ± 0.3	
10^{-7} M	10^{-7} M	3	0.2 ± 0.05	
10^{-6} M	10^{-6} M	3	0.05 ± 0.02	
10^{-5} M	10^{-5} M	3	0.09 ± 0.05	

* Determined 17 sec after FMLP and $C5a_{des\ arg}$ addition. FMLP (10^{-10} M–10^{-5} M) did not significantly stimulate cAMP levels ($P > 0.1$), whereas $C5a_{des\ arg}$ did ($P < 0.005$).

within 1 min following stimulation of human granulocytes (Fig. 4). F-met-leu-phe induced crawling movements, directional locomotion as well as a cAMP peak in human neutrophils (Table 3, Fig. 1) but none of these effects in equine neutrophils (Tables 1 and 3). Yet $C5a_{des\ arg}$ produced all these effects in equine neutrophils (Tables 1 and 3).

DISCUSSION

Earlier experiments have shown that chemotactic stimulation of neutrophils produced a transient increase in cAMP levels [9, 10, 13] and that a sustained increase in cAMP levels inhibited chemotaxis [1–5]. The present experiments were performed in order to obtain further information on the biological significance of the short transient cAMP increase observed within the first minute following chemotactic stimulation. They show that the cAMP peak is indeed closely associated with the chemotactic response. The cAMP peak seems to occur before or concomitantly with the beginning extension of lamellipodia towards the source of the attractant after the initial stimulation [19]. We only observed

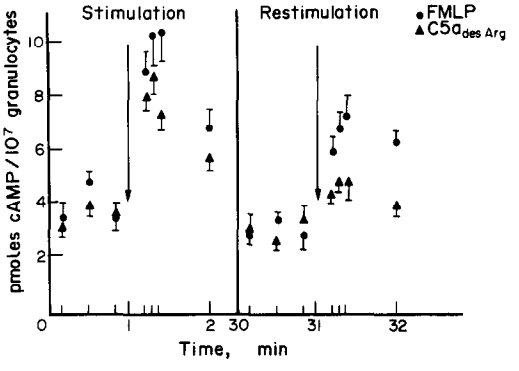

Fig. 2. cAMP increase after exposure of neutrophils to $C5a_{des\ arg}$ or f-met-leu-phe (FMLP) for 1 min and restimulation with the same cytotaxin. Human neutrophils were suspended in Gey's solution containing 2% HSA. First and second stimulation were done with either $C5a_{des\ arg}$ (final concentration 6.7×10^{-8} M) or FMLP (final concentration 5×10^{-8} M). Data represent the mean of 8 (▲) and 7 (●) individual experiments. A significant increase in cAMP levels was induced by $C5a_{des\ arg}$ as by FMLP after the first ($P < 0.005$) and second stimulation ($P < 0.005$).

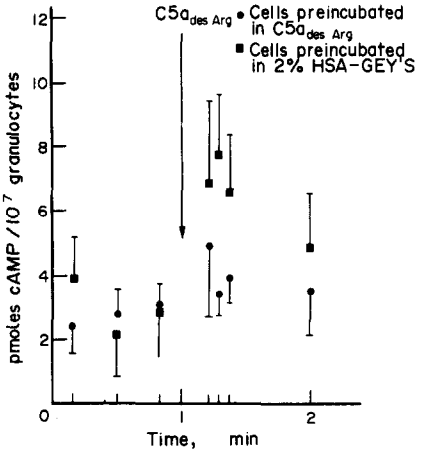

Fig. 3. cAMP levels after restimulation of partially chemotactic deactivated neutrophils with $C5a_{des\ arg}$. Human neutrophils suspended in Gey's solution containing 2% HSA were stimulated with $C5a_{des\ arg}$ (final concentration 6.7×10^{-8} M) after a preincubation in 2% HSA or $C5a_{des\ arg}$ (final concentration 3.3×10^{-7} M) for 30 min. Data represent the mean of 3 individual experiments each. In contrast to cells preincubated in 2% HSA–Gey's ($P < 0.005$), cells preincubated in $C5a_{des\ arg}$ showed no significant increase in cAMP level after a further $C5a_{des\ arg}$ stimulation ($P > 0.05$).

Table 2. Neutrophil migration after exposure to f-met-leu-phe (FMLP) or $C5a_{des\ arg}$ for 1 or 30 min

Test solution	Medium for incubation	Medium and time for preincubation	Incubation time (hr)	Per cent neutrophils migrated (± S.D.)
2% HSA–Gey's	2% HSA–Gey's	2% HSA–Gey's (1 min)	1	0.09 ± 0.08
FMLP, 5×10^{-8} M	2% HSA–Gey's	2% HSA–Gey's (1 min)	1	3.7 ± 2.3
$C5a_{des\ arg}$, 6.7×10^{-8} M	2% HSA–Gey's	2% HSA–Gey's (1 min)	1	17.4 ± 4.6
2% HSA–Gey's	2% HSA–Gey's	FMLP, 5×10^{-8} M (1 min)	1	0.02 ± 0.04
FMLP, 5×10^{-8} M	2% HSA–Gey's	FMLP, 5×10^{-8} M (1 min)	1	5.7 ± 0.6
2% HSA–Gey's	2% HSA–Gey's	$C5a_{des\ arg}$, 6.7×10^{-8} M (1 min)	1	0.02 ± 0.02
$C5a_{des\ arg}$, 6.7×10^{-8} M	2% HSA–Gey's	$C5a_{des\ arg}$, 6.7×10^{-8} M (1 min)	1	19.2 ± 6.0
2% HSA–Gey's	2% HSA–Gey's	2% HSA–Gey's (30 min)	1	0.2 ± 0.1
$C5a_{des\ arg}$, 6.7×10^{-8} M	2% HSA–Gey's	2% HSA–Gey's (30 min)	1	26.0 ± 2.8
2% HSA–Gey's	2% HSA–Gey's	$C5a_{des\ arg}$, 3.3×10^{-7} M (30 min)	1	0.2 ± 0.2
$C5a_{des\ arg}$, 6.7×10^{-8} M	2% HSA–Gey's	$C5a_{des\ arg}$, 3.3×10^{-7} M (30 min)	1	11.0 ± 1.5
2% HSA–Gey's	2% HSA–Gey's	2% HSA–Gey's (30 min)	3	7.8 ± 1.4
2% HSA–Gey's	2% HSA–Gey's	$C5a_{des\ arg}$, 3.3×10^{-7} M (30 min)	3	7.9 ± 1.9

Table 3. Effect of f-met-leu-phe (FMLP), $C5a_{des\ arg}$, immunoglobulin (SGG) and human serum albumin (HSA) on crawling movements of neutrophil granulocytes

Medium	Per cent neutrophils showing crawling movements: Human neutrophils *	Human neutrophils †	Equine neutrophils
2% HSA–Gey's	1	5.7	
FMLP, 5×10^{-8} M	90	82.0	
SGG 2%		76.0	
2% HSA–Gey's			3.8
FMLP, 10^{-9} M			9.3
5×10^{-8} M			4.9
10^{-7} M			3.9
10^{-6} M			3.6
10^{-5} M			5.8
2% HSA–Gey's			0
$C5a_{des\ arg}$, 3.3×10^{-10} M			25
3.3×10^{-9} M			93
3.3×10^{-8} M			81.8
1.1×10^{-6} M			71

*†, different series of experiments.

a cAMP peak in cells that also produced a chemotactic response to the particular stimulant used. Non-chemotactic analogues or chemotactic factors acting on cells which fail to respond chemotactically had no such effect. Gly-his-gly may have a small chemokinetic effect but was not chemotactic in our hands nor does it induce a peak of cAMP [24]. Also the non-chemotactic f-met-peptide analogue, CBZ-phe-met, does not produce a transient cAMP increase. This suggests that binding of a non-chemotactic ligand to the receptors is not sufficient to induce the cAMP increase. Equine neutrophils which

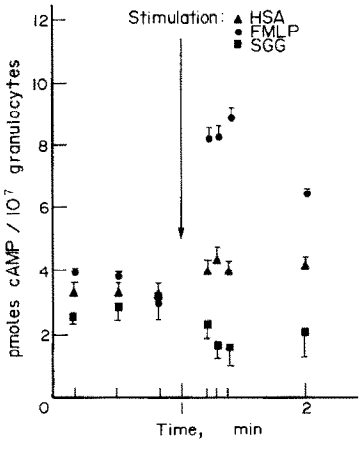

Fig. 4. Effect of immunoglobulin (SGG) on the cAMP level of neutrophils. Human neutrophils suspended in (a) Gey's solution were stimulated with HSA (final concentration 1%), and (b) Gey's solution containing 2% HSA were stimulated with FMLP (final concentration 5×10^{-8} M) or SGG (final concentration 2%). Data represent the mean of 7 (▲), 3 (■) and 16 (●) individual experiments. HSA ($P > 0.1$) as well as SGG did not significantly increase cAMP levels.

had about 60 times fewer receptors as human neutrophils [25, 26] failed to show chemotaxis and a cAMP peak in response to f-met-leu-phe (Table 1).

The fact that all chemotactic factors tested produce a transient cyclic AMP peak is compatible with the hypothesis that there is a causal relationship between chemotaxis and cAMP peak. There was, however, no quantitative relationship between the magnitude of the chemotactic response and the cAMP peak. Thus, the peak can not be used as a biochemical measure for directional locomotion induced by cytotaxins. This does not necessarily exclude a causal relationship between chemotaxis and cAMP.

Cytotaxins may or may not produce a variety of reactions other than chemotaxis such as chemokinesis, exocytosis, a metabolic burst and others. Therefore, the generation of cAMP peak does not necessarily imply that the cAMP is exclusively associated with chemotactic orientation. It could equally well be associated with any of the other activities taking place in response to cytotaxins. We have therefore investigated, whether there is a correlation between the induction of crawling movements and chemokinesis in general on the one hand and the appearance of the cAMP peak on the other hand. One means to produce granulocyte chemokinesis is to modulate adhesion to the substratum, e.g. by addition of serum albumin [28]. The present experiments confirm previous results showing that HSA does not produce a cAMP peak [9].

Positive chemokinesis can also reflect increased motility due to the transformation of spherical cells into cells that perform crawling movements resulting in locomotion after appropriate contact with the substratum [23]. It seems reasonable to assume that increased phosphorylation of contractile structures occurs in the course of this type of activation of the locomotor apparatus. Evidence for increased phosphorylation of myosin heavy chains following chemotactic stimulation has been presented in *Dictyostelium discoideum* [29]. We have therefore investigated whether the induction of crawling movements is associated with the generation of a cAMP peak. The evidence available at present suggests that chemotactic factors such as $C5a_{des\ arg}$ or f-met-leu-phe as well as non-chemotactic factors such as SGG can induce crawling-like movements in neutrophil granulocytes [23]. The immunoglobulin preparation tested induced crawling movements in floating neutrophils as efficiently as f-met-leu-phe (Table 3). In contrast to f-met-leu-phe, SGG has no detectable chemotactic activity [22, 30] and produces no cAMP peak (Fig. 4). This shows that the short transient generation of cAMP is no prerequisite for the induction of crawling movements. Thus, the hypothesis that cAMP might promote phosphorylation of contractile proteins is not supported by these results. It seems more attractive to speculate that cAMP has some other function in chemotaxis rather than chemokinesis, e.g. to promote phosphorylation or methylation of other substrata in the cell membrane. Evidence for cyclic nucleotide-dependent regulation of phospholipid methylation during chemotaxis has been presented in *Dictyostelium discoideum* [31].

The present study shows that the generation of the cAMP peak is closely correlated with chemotaxis but not with chemokinesis. The relationship between the cAMP peak and exocytosis will be analysed in a forthcoming study.

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