Effects of β -adrenergic receptor activation, cholera toxin and forskolin on human natural killer cell function

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Membranes from highly purified natural killer (NK) cells were ADP-ribosylated by treatment with cholera toxin (CTX). CTX resulted in a single band of specific ^{32}P incorporation at M_r 43 600. CTX treatment of intact NK cells caused a 9-fold increase in cyclic AMP (cAMP) concentrations. Pretreatment of NK cells with CTX diminished their ability to lyse K562 tumour cells by up to 79%. Forskolin treatment elevated NK cell cAMP levels 8-fold and decreased lysis of K562 cells by up to 45%. Adrenaline and isoprenaline (isoproterenol) both inhibited lysis of K562 cells by approx. 35% and elevated cAMP by at least 2.5-fold, and their inhibition of lysis was reversed by propranolol. These data suggest that the stimulatory guanine-nucleotide-binding protein G_s coupled to β -adrenergic receptors is involved in transducing signals which inhibit NK cell lysis of tumour cells. CTX and forskolin also diminish the ability of NK cells to bind K562 cells (binding is necessary for lysis). This suggests that the NK-cell receptor(s) for the tumour cell may be altered as a consequence of cAMP-mediated events or by activation of G_s .

INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes which are cytotoxic to certain susceptible tumour cells and which also demonstrate anti-microbial activity [1–3]. The molecular signal transduction mechanisms utilized in the regulation of the NK-cell cytotoxic response (both activation and inhibition) are not well defined. It has been demonstrated in mixed lymphocyte populations that increased cyclic AMP (cAMP) levels are inhibitory to NK-cell lysis of tumour cells [4–8]. However, due to the large percentage of non-NK cells in these lymphocyte preparations, it is not clear if the inhibition of NK cytotoxicity is due to cAMP elevation in the NK cells or if it is an indirect effect mediated by the consequences of cAMP elevation in one or more of the contaminating cell types.

A signal-dependent activator of adenylate cyclase in many cell types is the α -subunit of the stimulatory guanine-nucleotide-binding protein (G-protein), $G_s\alpha$ [9,10]. The ADP-ribosylation of $G_s\alpha$ by cholera toxin (CTX) produces the form of $G_s\alpha$ which is able to activate adenylate cyclase [9,11,12]. CTX treatment of impure NK cells has been shown to inhibit NK-cell cytotoxicity [13,14]. However, since the effect of CTX was not studied in a highly purified population of NK cells, it was not known whether the effects of CTX were due to activation of a $G_s\alpha$, and a subsequent elevation of intracellular cAMP, in NK cells or to a secondary effect based on CTX affecting a $G_s\alpha$ in some other cell type. In order to study the involvement of G_s in the transduction of inhibitory signals in NK cells, we use here highly purified NK cell populations ($85 \pm 5\%$ NK).

NK cytotoxicity is inhibited in these preparations by treatment with CTX or forskolin, both of which elevate intracellular cAMP. Both adrenaline and isoprenaline (isoproterenol) inhibit the cytotoxicity of these NK cells as well as elevating cAMP. The effects of isoprenaline are blocked by propranolol. Moreover, there is a 43 600- M_r molecule, present in the membranes of NK cells, which is a substrate for ADP-ribosylation by CTX. The M_r and toxin-sensitivity of this molecule correspond to those of $G_s\alpha$ [9,10]. We also examined the effects of increased cAMP on the binding of NK cells to K562 cells and on the ability of NK cells to release their cytotoxic granules.

MATERIALS AND METHODS

Isolation of human NK cells

Heparin-treated blood from healthy human donors was applied to Ficoll-Hypaque (1.077 g/ml) (Sigma, St. Louis, MO, U.S.A. and Winthrop-Breon Laboratories, New York, NY, U.S.A.) and centrifuged at 550 g for 30 min [15]. Mononuclear cells were collected from the Ficoll-Hypaque and washed twice (250 g , 10 min) with phosphate-buffered saline (PBS; 10 mM-phosphate/2.7 mM-KCl/140 mM-NaCl). The cells were then suspended in complete RPMI 1640 medium (Whittaker M.A. Bioproducts, Walkersville, MD, U.S.A.) supplemented with 10% heat-inactivated (56 °C for 30 min) human AB serum (Whittaker M.A. Bioproducts) or bovine calf serum (Hyclone, Logan, UT, U.S.A.), 2 mM-L-glutamine and 50 μg of gentamicin/ml (Whittaker M.A. Bioproducts). Non-adherent cells were prepared by incubating the cells in glass Petri dishes followed by passage through a nylon wool column [16]. Highly purified NK cells were then prepared by a 'panning' procedure [3]. Briefly, the non-adherent cells were centrifuged at 250 g , and the pellet was suspended in approx. 0.5 ml of complete medium and incubated with anti-CD8 (1.25 $\mu\text{g}/10^8$ cells), anti-CD4 (2.5 $\mu\text{g}/10^8$ cells) and anti-CD3 (5 $\mu\text{g}/10^8$ cells) monoclonal antibodies (Becton-Dickinson, Mountain View, CA, U.S.A.) for 15–30 min at 4 °C. These monoclonal antibodies are to cell-surface molecules on T cells, which are the major non-NK-cell type in the non-adherent cell population. The antibody-treated cells were suspended in 10–15 ml of complete medium, applied to plastic Petri dishes coated with goat anti-(mouse IgG) (TAGO Inc., Burlingame, CA, U.S.A.) and incubated at 4 °C for 15–30 min. Antibody-coated cells adhered to the Petri dish. The unbound cells were collected and washed once with RPMI 1640. The panning procedure was repeated at least once. The unbound cells were suspended in complete medium at a concentration of 1×10^6 cells/ml and stored at 37 °C, in air/ CO_2 (19:1). This panning procedure was unpredictable in its efficiency of removal of T cells. A more reliable method for removing T cells from the non-adherent cells was to rosette them with sheep red blood cells (Colorado Serum, Denver, CO, U.S.A.) [17]. Non-adherent cells were washed once with PBS (250 g , 10 min) and suspended in

Abbreviations used: NK cells, natural killer cells; cAMP, cyclic AMP; G_s , stimulatory guanine-nucleotide-binding protein (G-protein); CTX, cholera toxin; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.

serum-free RPMI at 1×10^7 cells/ml. Sheep red blood cells were washed three times with PBS and 1 ml of these packed cells was suspended in 9 ml of serum-free RPMI. The non-adherent cell suspension was mixed with the sheep red blood cell suspension at a ratio of 2:1 (v/v). The mixture was incubated at 37 °C for 15 min and then the cells were pelleted (250 g, 5 min) and incubated at 4 °C for 45 min. The pellet was then resuspended in the supernatant and layered on to Ficoll-Hypaque (1.077 g/ml; 10 ml of Ficoll-Hypaque per 15 ml of rosetted cell suspension) and centrifuged at 550 g. The non-rosetting cells which floated on Ficoll-Hypaque were collected, washed twice with PBS and stored at 37 °C in air/CO₂ (19:1) in complete medium. Cells prepared in this way were $85 \pm 5\%$ pure NK cells as determined by fluorescence microscopy using phycoerythrin-conjugated anti-CD14 antibody (Becton-Dickinson).

Cytotoxicity assay

NK cytotoxicity was measured using a ⁵¹Cr release assay [3]. The target cell in all cytotoxicity assays was the NK-susceptible K562 tumour cell line. K562 cells were incubated with ⁵¹Cr (New England Nuclear, Boston, MA, U.S.A.) in 2 ml of bovine calf serum ($100 \mu\text{Ci}/1 \times 10^6$ cells) for 1.5 h at 37 °C in air/CO₂ (19:1). The cells were then washed twice with complete medium.

NK (effector) cells ($2.5 \times 10^5/100 \mu\text{l}$ for a 25:1 ratio) were added to the wells of conical-bottom microtitre plates (Linbro Chemical Co., Hamden, CN, U.S.A.). The effectors were diluted to give ratios of 12.5:1 ($1.25 \times 10^5/100 \mu\text{l}$) and 6.25:1 ($6.25 \times 10^4/100 \mu\text{l}$); each ratio was tested in triplicate. Targets were added (1×10^4 cells/ $100 \mu\text{l}$) to each well, and the plate was centrifuged at 50 g for 3 min and incubated for 4 h at 37 °C (air/CO₂, 19:1). After incubation a $100 \mu\text{l}$ portion of the supernatant was counted for radioactivity for 1 min in a γ -radiation counter. The specific lysis (%) was determined as follows: $100 \times [(\text{test c.p.m.} - \text{spontaneous c.p.m.})/(\text{maximum c.p.m.} - \text{spontaneous c.p.m.})]$. Maximum release was produced by adding the $100 \mu\text{l}$ of target cells to wells containing $100 \mu\text{l}$ of 20% Triton X-100.

Degranulation assay

Degranulation of the NK cells was determined by measuring release of the enzyme β -hexosaminidase, as this enzyme has been shown to be released with the secretory granules of mast cells [18]. Cells [$(1-2) \times 10^6$] were incubated in $210 \mu\text{l}$ of 10 mM-Hepes (pH 7.2)/5 mM-glucose/1.8 mM-CaCl₂/0.7 mM-MgSO₄/0.13% NaHCO₃/0.05% BSA at 37 °C in air/CO₂ (19:1). The incubation was stopped by adding $200 \mu\text{l}$ of cold PBS and placing the tubes on ice. The tubes were then centrifuged at approx. 800 g at 4 °C for 10 min. β -Hexosaminidase activity was measured by incubating a $250 \mu\text{l}$ portion of the supernatant with 4 mM-*p*-nitrophenyl β -D-2-acetamido-2-deoxyglucopyranoside/0.04 M-citrate buffer brought to pH 4.5 with 0.2 M-Na₂-PO₄. The product of the enzymic reaction, *p*-nitrophenol, was measured at 495 nm. Maximal β -hexosaminidase release was determined by treating the cells with 0.5% Triton X-100. Results are presented as percentages of maximal β -hexosaminidase release.

Phorbol 12-myristate 13-acetate (PMA; 20 nM) and calcium ionophore A23187 (250 nM) were used to stimulate maximal degranulation of the NK cells. It has already been shown that PMA and A23187 can stimulate release of NK cytotoxic factors (cytotoxic granules) [19].

cAMP assay

The amount of cAMP present in NK cells after various treatments was determined by radioimmunoassay (RIANEN cAMP ¹²⁵I r.i.a. kit; New England Nuclear) and by a cAMP-binding protein assay (Diagnostic Products Corporation, Los

Angeles, CA, U.S.A.). Control and treated NK cells were incubated at $(2.5-5) \times 10^6/\text{ml}$ in complete medium containing 0.5 mM-3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor) for 1-2 h at 37 °C in air/CO₂ (19:1). The cells were pelleted and cAMP was extracted by the addition of 1 M-HCl/ethanol (1:100, v/v) [4]. After addition of the ethanol the tubes were vortex-mixed and then centrifuged at 800 g to pellet the insoluble material. The supernatant was evaporated under N₂ and the dried material was resuspended in assay buffer. cAMP levels in the samples were determined from standard curves.

Conjugation of NK cells to target cells

The percentage of target cells with NK cells bound [20] was determined for three effector cell/target cell ratios; 25:1, 12.5:1 and 1:1. A total of 2.5×10^4 target cells was incubated with the appropriate number of control or treated effector cells at 37 °C in air/CO₂ (19:1) for 1-2 h. The cells were viewed under a light microscope and the number of target cells with NK cells bound to their surface was determined, relative to total target cells.

NK-cell membrane preparation and ADP-ribosylation

NK-cell membranes were prepared from purified NK cells using the procedure described by Gilmore & Weiner [21]. The cells were twice suspended in 0.9 M-NaCl and centrifuged at 800 g. The washed cells were then resuspended in 25 mM-Na-Hepes (pH 8.0)/120 mM-NaCl/1 mM-EDTA and homogenized by approx. 20 strokes in a glass Potter-Elvehjem homogenizer. The homogenized cells were pelleted at 800 g and the membranes were stored at -80 °C. CTX (Sigma) was preincubated with 50 mM-dithiothreitol at 32 °C for 30 min in order to disassociate the enzymically active A1 subunit [12]. Membranes were incubated for 2 h at 32 °C in 80 mM-potassium phosphate (pH 7.5)/10 mM-MgCl₂/10 mM-thymidine/1 mM-ATP/ $10 \mu\text{M}$ -³²P]NAD⁺ with or without CTX (total volume $120 \mu\text{l}$). The reaction was stopped by the addition of 1 ml of ice-cold 20% trichloroacetic acid and the incubation mixture was centrifuged at 800 g, 4 °C for 20 min. The pellet was washed with 1 ml of cold acetone and centrifuged at 800 g, 4 °C for 20 min. The pellet was resuspended in sample buffer (without heating) and subjected to SDS/PAGE (10% gel) using the method of Laemmli [22]. Autoradiography was carried out on dried gels (which had been stained with Coomassie Blue) using Kodak XAR5 film.

RESULTS

Effect of CTX on the cytotoxicity of NK cells

It is known that CTX catalyses the ADP-ribosylation of G_s α , resulting in activation of adenylate cyclase [9]. If G_s is involved in the transduction of inhibitory signals in NK cells, then CTX treatment of NK cells should inhibit their cytotoxicity. Fig. 1 shows the effect of CTX ($0.5 \mu\text{g}/\text{ml}$) treatment on the ability of NK cells to lyse K562 target cells. There was significant inhibition of K562 lysis at all effector cell/target cell ratios studied. Specific lysis was inhibited by 79% at an effector cell/target cell ratio of 25:1. The β subunit of CTX, which alone does not ADP-ribosylate G_s α , had no effect on NK cytotoxicity (results not shown).

Effects of forskolin on the cytotoxicity of NK cells

Raising cAMP by another agent (which does not activate G_s α) should also result in inhibition of specific lysis if increased cAMP itself is at least partially responsible for the inhibition caused by CTX. The cytotoxicity assay was carried out in the presence of $10 \mu\text{M}$ -forskolin (Fig. 2), which raises cAMP by direct stimulation of adenylate cyclase [23]. Specific lysis of K562 targets was inhibited at all effector cell/target cell ratios. The extent of

Fig. 1. Effect of CTX treatment on specific lysis of K562 cells by NK cells at three NK (effector) cell/K562 (target) cell ratios

●, Control NK cells. NK cells (○) were pretreated with 0.5 μ g of CTX/ml in complete medium, at a concentration of 1×10^6 cells/ml, for 30 min at 37 °C. CTX-containing medium was removed by pelleting the cells at 150 *g* prior to assaying for cytotoxicity. Values are the means $\pm$ S.D. from at least three separate experiments (triplicate determinations) using NK cells from three different donors. Significant differences at the various effector cell/target cell ratios were as follows: 25:1, $P < 0.0005$; 12.5:1, $P < 0.005$; 6:1, $P < 0.025$.

Fig. 2. Effect of 10 μ M-forskolin on specific lysis of K562 cells by NK cells at three NK (effector) cell/K562 (target) cell ratios

●, Control NK cells; ○, 10 μ M-forskolin was present during assay. Values are the means $\pm$ S.D. from three experiments (triplicate determinations) using NK cells from three different donors. Significant differences from control at the various effector cell/target cell ratios were as follows: 25:1, $P < 0.025$; 12.5:1, $P < 0.01$; 6:1, $P < 0.01$.

inhibition at ratios of 25:1 and 12.5:1 (on average 44%) was not as great as that seen in CTX-treated cells.

Effects of adrenaline and isoprenaline on NK cytotoxicity

We next looked at the effects on cytotoxicity of ligands for the β -adrenergic receptor, a natural activator of G_s . Adrenaline (1 μ M) (Fig. 3) inhibited specific lysis by up to 33%, and 1 μ M-isoprenaline (a selective β -receptor agonist) (Fig. 4) inhibited specific lysis of K562 cells by up to 39%. The presence of 1 μ M-

Fig. 3. Effect of 1 μ M-adrenaline on specific lysis of K562 cells at three NK (effector) cell/K562 (target) cell ratios

●, Control cells; ○, 1 μ M-adrenaline was present during assay. Values are from a representative experiment (triplicate determinations). At least three separate experiments were done.

Fig. 4. Effects of 1 μ M-isoprenaline, and 1 μ M-isoprenaline plus 1 μ M-propranolol, on specific lysis of K562 cells at three NK (effector) cell/K562 (target) cell ratios

●, Control cells; ○, 1 μ M-isoprenaline present during assay; Δ , 1 μ M-isoprenaline plus 1 μ M-propranolol present during assay. Values are from a representative experiment (triplicate determinations). Three separate experiments were done.

propranolol blocked the inhibition by both adrenaline (results not shown) and isoprenaline (Fig. 4).

Effects of CTX, forskolin, adrenaline and isoprenaline on intracellular cAMP levels in NK cells

ADP-ribosylation of $G_s\alpha$ in NK cells by CTX increased intracellular cAMP by roughly 9-fold (Table 1). Similarly, 10 μ M-forskolin caused an 8-fold increase in NK-cell cAMP levels, 1 μ M-adrenaline increased cAMP by 2.5-fold and 1 μ M-isoprenaline resulted in a 2.8-fold increase (Table 1).

CTX treatment of NK cell membrane ADP-ribosylates an approx. 43600- M_r substrate

Fig. 5(a) shows an autoradiogram of a SDS/PAGE [10% (w/v) polyacrylamide gels] of NK-cell membranes which were incubated with [32 P]NAD $^{+}$, with and without CTX. The CTX-treated membranes showed a labelled band at an M_r of approx.

Table 1. cAMP content of NK cells after CTX, forskolin or adrenaline treatment

NK cells were routinely incubated with CTX, forskolin or adrenaline for 1–2 h. Results are from experiments carried out on NK-cell preparations from at least three different donors. Numbers in parentheses indicate numbers of separate experiments (duplicate determinations).

Treatment	cAMP (pmol/10 ⁶ cells)
Control	0.61 ± 0.20 (3)
Forskolin (10 μ M)	5.00 ± 2.90 (3)
Control	0.52 ± 0.13 (9)
CTX (0.5 μ g/ml)	4.58 ± 1.8 (9)
Control	0.55 ± 0.06 (3)
Adrenaline (1 μ M)	1.40 ± 0.43 (3)
Control	0.33 ± 0.02 (2)
Isoprenaline (1 μ M)	0.92 ± 0.38 (2)

Fig. 5. ADP-ribosylation of NK-cell membranes

(a) Autoradiogram after SDS/PAGE [10% (w/v) polyacrylamide] of [³²P]NAD⁺-treated NK-cell membranes: lane 1, control; lane 2, CTX-treated. (b) Autoradiogram after SDS/PAGE of [³²P]NAD⁺-treated NK-cell membranes: lane 1, membranes of cells pretreated with CTX and then incubated with [³²P]NAD⁺ and CTX; lane 2, membranes of cells with no CTX pretreatment which were incubated with [³²P]NAD⁺ and CTX. *M_r* markers were: BSA, 66 000; ovalbumin, 45 000; glyceraldehyde-3-phosphate dehydrogenase, 36 000; carbonic anhydrase, 29 000; trypsinogen, 24 000; soybean trypsin inhibitor, 20 100. The autoradiograms are from representative experiments; the lane 2 in both (a) and (b) shows a radioactive band at *M_r* 43 600. The results were repeated in NK-cell membranes from three different donors.

43 600, which corresponds roughly to the *M_r* of G_α in other cell types (approx. 45 000 [9]). The labelled band at approx. *M_r* 37 000 (Fig. 5a) was seen in both the presence and the absence of CTX. When membranes from cells that were pretreated with CTX (0.5 μ g/ml) were incubated with [³²P]NAD⁺ in the presence of CTX, the 43 600-*M_r* band was not labelled (Fig. 5b).

Effect of CTX treatment on the release of granules from NK cells

It has been shown that stimulation of protein kinase C by PMA and A23187 will release the cytotoxic factors from NK cells [19]. Table 2 shows that CTX-treated NK cells degranulated to the same extent as control cells when protein kinase C was

Table 2. Effect of CTX on release of NK-cell granules

Results are means ± S.D. of duplicate determinations from two or three different NK cell preparations.

Stimuli for release	Pretreatment of NK cells	Release of β -hexosaminidase (% of maximal)
PMA (20 nM) + A23187 (250 nM)	Control	33 ± 5
PMA (20 nM) + A23187 (250 nM)	CTX (0.5 μ g/ml)	32 ± 6
CTX (0.5 μ g/ml)	Control	-0.1 ± 1

stimulated by PMA and A23187. This indicates that protein kinase C is not being inhibited dramatically by elevated cAMP. It also shows that control and CTX-treated cells release the same percentage of their total β -hexosaminidase when stimulated by PMA and A23187, indicating that releasable-granule number is not affected by increased cAMP. Table 2 also indicates that preincubation with 0.5 μ g of CTX/ml does not cause release of granules.

Effect of CTX and forskolin on effector–target conjugation

The ability of NK cells to conjugate with the K562 target cells was compared among CTX-treated, forskolin-treated and control cells (Fig. 6). CTX treatment reduced the fraction of target cells with NK cells bound to them by roughly 40% at all effector cell/target cell ratios examined. Forskolin treatment also significantly inhibited the ability of NK cells to bind targets at the 12.5:1 and 1:1 ratios, but to a lesser extent than did CTX. This inhibition of conjugate formation would contribute to the decreased cytotoxicity of NK cells which have increased intracellular cAMP.

Fig. 6. Effect of CTX and forskolin on NK cell conjugation to K562 target cells at three effector cell/target cell ratios

●, NK cells were pretreated with 0.5 μ g of CTX/ml in complete medium, at a concentration of 1×10^6 cells/ml, for 30 min at 37 °C. CTX-containing medium was removed by pelleting the cells at 150 *g* prior to assaying for conjugation. ○, Forskolin (10 μ M) was present during the conjugation assay. Values are the means ± S.D. from experiments (duplicate determinations) using NK cells from three different donors.

DISCUSSION

The data presented here suggest that a G_s is involved in transmembrane signalling in human NK cells. β -Adrenergic receptors appear to constitute at least one receptor type which is linked to this G_s . Direct activation of G_s by CTX inhibits the cytotoxicity of NK cells while raising cAMP levels by 9-fold. Forskolin elevates cAMP (8-fold) by direct stimulation of adenylate cyclase, and also inhibits NK cytotoxicity. The β -adrenergic receptor agonists adrenaline and isoprenaline inhibit NK cytotoxicity as well as raising cAMP, and the inhibition of cytotoxicity by both agents is blocked by propranolol. Finally, CTX treatment of NK-cell membranes shows ADP-ribosylation of a 43 600- M_r substrate; this M_r is similar to that of $G_s\alpha$ in other cells [9,10].

Although cAMP (and agents that elevate it) has been shown to be inhibitory to NK-cell cytotoxicity [4-8,13,14] the data presented in this study are the first utilizing highly purified populations of NK cells, thus establishing that the agents tested are probably having their effect on the NK cells and not on another cell population which secondarily inhibits NK function.

The mechanism(s) by which G-protein-mediated cAMP elevation inhibits cytotoxicity were also investigated, by examining the effector cell-target cell conjugation as well as certain post-conjugation steps of NK cytotoxicity. Fig. 6 shows that a significant inhibition of conjugation occurs when cAMP is elevated by either CTX or forskolin treatment. This inhibition of conjugate formation could account for some or all of the inhibition observed with CTX.

We also show that protein kinase C activation (which is necessary for the release of cytotoxic granules from NK cells [19]) is not blocked when cAMP is elevated by CTX treatment of NK cells. However, cAMP has been shown to inhibit phosphoinositide hydrolysis in an NK clone [4], which indicates that phospholipase C inhibition may be a consequence of elevated cAMP.

Thus the effect of decreased conjugation may only be one of the inhibitory effects of elevated cAMP on NK-cell cytotoxicity. The extent of inhibition by forskolin on both cytotoxicity and conjugation was not as great as with CTX, despite the fact that they caused very similar increases in NK-cell cAMP. One explanation for these results is that there are cAMP-independent effects of CTX occurring in NK cells, as have been seen in other cell types [24,25].

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