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Effects of pertussis toxin treatment on human natural killer cell function

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SUMMARY

Membranes from highly purified natural killer (NK) cells were ADP ribosylated by treatment with pertussis toxin (PTX). PTX treatment resulted in a single band of ^{32}P incorporation at M_r 41,600. PTX treatment of NK cells diminished their ability to lyse K562 tumour cells by about 50%. However PTX treatment had no measurable effect on cAMP levels in NK cells. PTX pretreatment also had no effect on the ability of target cells to induce phosphoinositide turnover or on the ability of the NK cells to conjugate with the K562 tumour cells. Movement toward the chemoattractants interleukin-2 (IL-2) and formylmethionylleucylphenylalanine (FMLP) was significantly inhibited indicating that a PTX substrate in NK cells may be involved in the transduction of signals which are involved in cell motility.

INTRODUCTION

Natural killer (NK) cells are large granular lymphocytes which play a role in natural, non-adaptive immunity. They are cytotoxic to certain susceptible tumour cells and were initially identified by this function.[1,2] In addition to this cytotoxic function NK cells also provide natural surveillance against some microbial infections, produce lymphokines, and have a regulatory function in haematopoeisis and the adaptive immune system.[3] Activation of phospholipase C (PLC) and a subsequent rise in intracellular calcium have been measured following NK cells binding to lysis-sensitive tumour cells.[4] However the molecular signal transduction mechanisms utilized in the regulation of the NK-cell cytotoxic response (which includes locomotion, tumour cell binding and cytotoxic granule release) are not well defined.

Guanine nucleotide binding proteins (G proteins) are responsible for transducing signals from a variety of cell surface receptors.[5] We have shown that Gs, the stimulatory G protein for adenylate cyclase, is present in NK cells and its activation results in an inhibition of NK cytotoxicity.[6]

Pertussis toxin (PTX) ADP ribosylates the alpha subunits of some classes of G proteins (Gα), and usually results in the inactivation of those Gαs that it affects.[5] In order to probe further the involvement of G proteins in the signal transduction of the cytotoxic response of NK cells we studied the effect of PTX treatment on the functions of a highly purified preparation of NK cells.

We show that NK cytotoxicity is inhibited by treatment with PTX by about 50%. Elevated cAMP levels in NK cells do not appear to be responsible for this inhibition as cAMP levels remained unchanged after PTX treatment. NK binding to tumour cells is unaffected by PTX treatment as is the release of cytotoxic granules. Stimulation of PLC in NK cells by lysis-sensitive tumour cells is also unaffected by PTX treatment. However, locomotion of NK cells toward the chemoattractants interleukin-2 (IL-2) and FMLP were significantly inhibited by PTX treatment. Finally, there is a 41,600 M_r molecule in the membranes of NK cells that is a substrate for ADP ribosylation by PTX.

Abbreviations: CTX, cholera toxin; cAMP, cyclic AMP; FMLP, formylmethionylleucylphenylalanine; Gi, inhibitory guanine-nucleotide-binding protein (G protein); IL-2, interleukin-2; NK, natural killer; PTX, pertussis toxin; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; SDS-PAGE, sodium-dodecyl-sulphate-polyacrylamide-gel electrophoresis.

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MATERIALS AND METHODS

Isolation of human NK cells

Buffy coats from a unit (450 ml) of blood were obtained from a local blood bank. The buffy coat was applied to Ficoll–Hypaque (1·077 g/ml) (Sigma, St Louis, MO and Winthrop-Breon Laboratories, New York, NY) and centrifuged at 800 ***g*** for 30 min[7] to remove contaminating red blood cells. Mononuclear cells were collected from the Ficoll–Hypaque and washed twice (250 ***g***, 10 min) with phosphate-buffered saline [PBS; 10 mM phosphate (pH 7·2)/2·7 mM KCl/140 mM NaCl]. The cells were then suspended in complete medium which consisted of RPMI-1640 (Sigma) supplemented with 10% heat-inactivated (56° for 30 min) bovine calf serum (BCS) (Hyclone, Logan, UT), 2 mM L-glutamine, 50 μg/ml gentamicin (Sigma), and 50 U penicillin G with 50 μg streptomycin/ml (Sigma). Non-adherent cells were prepared by incubating the cells in glass Petri dishes (150 × 15

mm).[8] The non-adherent cells were rosetted with sheep red blood cells (SRBC) (Colorado Serum, Denver, CO)[9] to remove T lymphocytes which are the major non-NK-cell type in the non-adherent cell population. Non-adherent cells were washed twice with PBS (250 *g*, 10 min) and suspended in serum-free RPMI-1640 at 1×10^7/ml. SRBC were washed three times with PBS and 1 ml of these packed cells was suspended in 9 ml of serum-free RPMI-1640. The non-adherent cell suspension was mixed with the SRBC suspension at a ratio of 2:1 (v/v). The mixture was incubated at 37° for 15 min and then the cells were pelleted (250 *g*, 10 min) and incubated at 4° 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 800 *g* for 30 min. The non-rosetting cells which floated on Ficoll–Hypaque were collected, washed twice with PBS and stored at 37° in air/CO_2 (19:1) in complete medium. The cells prepared in this way gave a highly purified population of NK cells. These cell preparations were greater than 97% $CD3^-$, approximately 90% $CD16^+$ and contained no detectable B cells. Phenotype was determined by fluorescence microscopy using phycoerythrin-conjugated anti-CD16, anti-CD3 and anti-CD19 antibodies (Becton Dickinson, San Jose, CA).

Cytotoxicity assay

NK cytotoxicity was measured using a ^{51}Cr release assay.[10] The target cell in all cytotoxicity assays was the NK-susceptible K562 (human chronic myelogenous leukaemia) cell line. K562 cells were incubated with ^{51}Cr (New England Nuclear, Boston, MA) in 1 ml of bovine calf serum (50 μCi/1 $\times 10^6$ cells) for 2 hr at 37° in air/CO_2 (19:1). The cells were then washed twice with complete medium.

NK (effector) cells ($2{\cdot}5 \times 10^5$/100 μl for a 25:1 ratio) were added to the wells of round-bottom microtitre plates (Costar, Cambridge, MA). The effectors were diluted to give ratios of 12·5:1 ($1{\cdot}25 \times 10^5$/100 μl) and 6·25:1 ($6{\cdot}25 \times 10^4$/100 μl); each ratio was tested in triplicate. Targets were added (1×10^4/100 μl) to each well, and the plate was centrifuged at 50 *g* for 3 min and incubated for 4 hr at 37° (air/CO_2, 19:1). After incubation the supernatant was collected using a harvesting press (Skatron, Sterling, VA) and counted for radioactivity for 1 min in a gamma radiation counter. Specific lysis (%) was determined as follows: 100 × [(test c.p.m. − spontaneous c.p.m.)/(maximum c.p.m. − spontaneous c.p.m.)]. Maximum release was produced by adding 100 μl of target cells to wells containing 100 μl of 10% Triton X-100.

cAMP assay

The amount of cAMP present in NK cells was determined by a cAMP-binding protein assay (Diagnostic Products Corporation, Los Angeles, CA). Control and treated NK cells were incubated at 2 to 5×10^6/ml in complete medium containing 0·5 mM 3-isobutyl-1-methylxanthine (phosphodiesterase inhibitor) for 1–2 hr at 37° in air/CO_2 (19:1). The cells were pelleted and cAMP was extracted by the addition of 1 ml 95% ethanol. After addition of the ethanol the tubes were vortex mixed and then centrifuged at 800 *g* to pellet the insoluble material. The supernatant was removed and evaporated under N_2 and the dried material was resuspended in assay buffer. cAMP levels in the samples were determined from standard curves.

NK cell membrane preparation and ADP ribosylation

NK-cell membranes were prepared from purified NK cells using the procedure described by Gilmore and Weiner.[11] The cells were twice suspended 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 passage through a 21-gauge needle (approximately 20×) followed by passage through a 25-gauge needle (approximately 20×). The homogenized cells were pelleted at 800 *g* and the membranes were stored at −80°. PTX (Sigma) was preincubated with 50 mM dithiothreitol at 32° for 30 min in order to disassociate the enzymatically active A subunit. Membranes were incubated for 2 hr at 32° in 75 mM Tris-HCl (pH 7·5)/10 mM $MgCl_2$/10 mM thymidine/1 mM ATP/10 μM [^{32}P]NAD with or without PTX (total incubation volume 120 μl). The reaction was stopped by the addition of 1 ml of ice-cold 20% trichloroacetic acid and centrifugation at 800 *g*, 4° for 20 min followed by suspension of the pellet in cold acetone (1 ml) and centrifugation at 800 *g*, 4° for 10 min (2×). The pellet was resuspended in sample buffer (without heating) and subjected to SDS–PAGE (10% gel) using the method of Laemmli.[12] Autoradiography was carried out on dried gels (which had been stained with Coomassie Blue) using Kodak XAR5 film.

Conjugation of NK cells to target cells

The percentage of target cells with NK cells bound[13] was determined for three effector cell/target cell ratios; 25:1, 12·5:1 and 1:1. A total $2{\cdot}5 \times 10^4$ target cells was incubated with the appropriate number of control or treated effector cells at 37° in air/CO_2 (19:1) for 1 hr. 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.

Degranulation assay

Release of granules from NK cells was determined by measuring release of the enzyme β-glucuronidase,[14] as this enzyme has been shown to be present in the granules of NK cells.[15] Cells (1 to 2×10^6) were incubated in 210 μl of 10 mM HEPES (pH 7·2)/5 mM glucose/1·8 mM $CaCl_2$/0·7 mM $MgSO_4$/0·13% $NaHCO_3$/0·05% bovine serum albumin (BSA) at 37° in air/CO_2 (19:1). The incubation was stopped by adding 200 μl of cold PBS and placing the tubes on ice. The tubes were then centrifuged at 800 *g* at 4° for 10 min. β-Glucuronidase activity was measured by incubating a 250-μl portion of the supernatant with 1 mM phenolphthalein glucuronic acid/0·04 mM citrate buffer brought to pH 4·5 with 0·2 M Na_2PO_4. The product of the enzymatic reaction, phenolpthalein, was measured at 552 nm. Maximal β-glucuronidase release was determined by treating the cells with 0·5% Triton X-100. Results are presented as percentage of maximal β-glucuronidase release.

NK-cell locomotion assay

NK-cell migration was evaluated in blind well chemotaxis chambers (Nucleopore Corp., Pleasanton, CA) using an 8 μ pore size cellulose nitrate membrane (Sartorius, Hayward, CA) to separate the upper and lower compartments.[16] NK cells 2×10^5/100 μl in RPMI-1640 containing 1% BSA were placed in the upper compartment of the chamber and IL-2 (100 U) or FMLP (10^{-5} M) in 100 μl of RPMI-1640 containing 1% BSA was placed in the lower compartment. The chambers were incubated for 2 hr at 37° and the membranes were then fixed

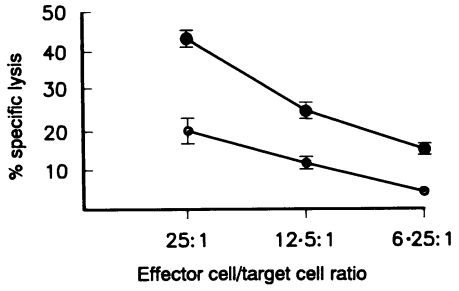

Figure 1. Effect of PTX treatment on specific lysis of K562 cells by NK cells at three NK (effector) cell/K562 (target) cell ratios. Control NK cells (●); pretreated with 2·5 μg/ml PTX in complete medium, at a concentration of 1×10^6 cells/ml, for 30 min at 37° (○). PTX-containing medium was removed by pelleting the cells at 150 *g* prior to assaying for cytotoxicity. Values are from a representative experiment, means ± SD from triplicate determinations. The results were repeated in NK cells from three different donors.

with formaldehyde, stained with haemotoxylin, and rinsed with isopropyl alcohol followed by xylene. Migration was evaluated by the leading front method[16] using the microscope micrometer. Using this method migration is measured as the distance travelled (into the membrane) by the leading three (or more) cells, in microns. Three different places on each membrane are viewed. Thus the number of microns migrated is that of nine or more cells from three representative portions of the membrane. The migration index was calculated by subtracting the migration in response to media alone from the migration in response to a chemotactic factor (either IL-2 or FMLP).

Phospholipase C assay

NK cells at a concentration of 2×10^6/ml in Hanks' balanced salt solution (HBSS) with 20 mM HEPES (pH 7·2)/1·3 mM $CaCl_2$/0·81 mM $MgSO_4$/4·2 mM $NaHCO_3$/5% BCS, were labelled with 1 μCi/ml of [^{3}H]inositol (New England Nuclear) for 16 hr. The cells were then washed twice with PBS and suspended at a concentration of 10×10^6/ml in serum-free HBSS. Two hundred microlitres of the cell suspension was used per assay tube and the total incubation volume was brought to 300 μl with HBSS. The assay was terminated by the successive addition of 60 μl 0·22 N HCl and 1·35 ml chloroform:methanol (1:2, v/v). Phases were separated by adding 450 μl chloroform and 450 μl water followed by centrifugation at 600 *g* for 15 min. A 1·3 ml aliquot of the upper phase was diluted and neutralized by the addition of 2 ml of 5 mM disodium tetraborate/30 mM sodium formate and 1 ml of water. The diluted sample was applied to a 0·5 ml AG1X8 (formate form, BioRad, Richmond, CA) column. The column was eluted with 10 ml of water, followed by 10 ml 5 mM $Na_2B_4O_7$/30 mM CHOONa, followed by 5 ml each of (1) 0·1 M CHOOH/0·2 M $CHOONH_4$, (2) 0·1 M CHOOH/0·5 M $CHOONH_4$ and (3) 0·1 M CHOOH/1·0 M $CHOONH_4$.[4,17] The last three buffers which eluted IP, IP_2 and IP_3 respectively were collected and the radioactivity was counted in a beta radiation counter.

RESULTS

Effect of PTX on the cytotoxicity of NK cells

Figure 1 shows the effect of PTX (2·5 μg/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 53% at an effector cell/target cell ratio of 25:1, 56% at a ratio of 12·5:1 and by 75% at the effector cell/target cell ratio of 6·25:1. This trend of increasing inhibition with decreasing effector cell/target cell ratio was reproducible. The B subunit of PTX, which alone does not ADP ribosylate Gα had no effect on NK cytotoxicity (results not shown).

Table 1. cAMP content of NK cells

Treatment*	cAMP (pmol/10^6 cells)
Control	0·22 ± 0·09 (3)†
PTX (2·5 μg/ml)	0·17 ± 0·05 (3)
CTX (0·5 μg/ml)	3·51 ± 0·36 (3)

* NK cells were routinely incubated with PTX or cholera toxin (CTX) for 1–2 hr.

† Values are the means ± SD of duplicate determinations from three different NK-cell preparations; number of separate experiments in parentheses.

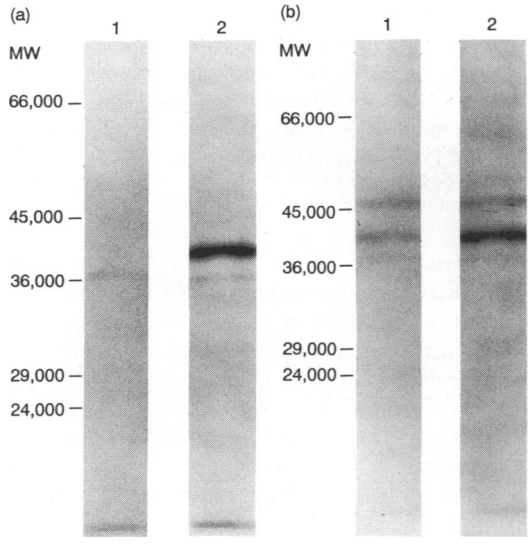

Figure 2. ADP ribosylation by PTX of NK-cell membranes. (a) Autoradiogram after SDS–PAGE [10% (w/v) polyacrylamide] of [^{32}P]NAD^+-treated NK-cell membranes: 1, control; 2, PTX treated. (b) Autoradiograms after SDS–PAGE of [^{32}P]NAD^+-treated NK-cell membranes: 1, membranes of cells pretreated with PTX and then incubated with [^{32}P]NAD^+ and PTX; 2, membranes of cells with no PTX pretreatment which were incubated with [^{32}P]NAD^+ and PTX. M_r markers were BSA, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000. The autoradiograms are from representative experiments; the right lane in both (a) and (b) shows a radioactive band at M_r 41,600. The results were repeated in NK-cell membranes from three different donors.

Effects of PTX on intracellular cAMP levels in NK cells

ADP ribosylation of the alpha subunit of the inhibitory G protein of adenylate cyclase (Gαi) by PTX could possibly cause

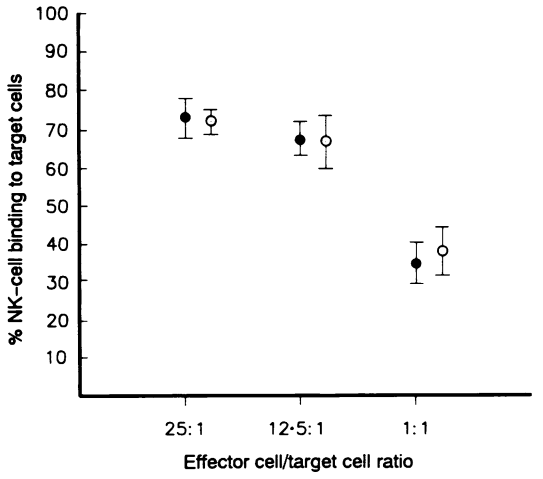

Figure 3. Effect of PTX treatment on NK-cell binding to K562 target cells at three effector cell/target cell ratios. Control cells (●); cells pretreated with 2·5 μg/ml PTX in complete medium at a concentration of 1×10^6 cells/ml, for 30 min at 37° (○). PTX-containing medium was removed by pelleting the cells at 150 *g* prior to assaying for binding. Values are the means ± SD from three experiments using three different NK-cell preparations (duplicate determinations).

Table 2. Effect of PTX on the release of NK-cell granules

Pretreatment of NK cells	Stimuli for release	Release of β-glucuronidase (% of maximal)
Control	PMA (20 nM) + A23187 (250 nM)	25 ± 5 (3)*
PTX (2·5 μg/ml)	PMA (20 nM) + A23187 (250 nM)	25 ± 2 (3)
Control	PTX (2·5 μg/ml)	1·3 ± 0·6 (3)

* Values are the means ± SD of duplicate determinations from three different NK-cell preparations; number of separate experiments in parentheses.

Table 3. Chemotaxis of NK cells toward IL-2 and FMLP

Treatment	Chemoattractant	Migration index
Control	IL-2	36 ± 5 (3)*
PTX (2·5 μg/ml)	IL-2	18 ± 5 (3)
Control	FMLP	22 ± 8 (3)
PTX (2·5 μg/ml)	FMLP	2 ± 2 (3)

* Values are the means ± SD of triplicate determinations from three different NK-cell preparations; number of separate experiments in parentheses.

Table 4. Inositol phosphate turnover in NK cells

Experiment 1

Treatment	Stimulus	% increase in 1,4 IP_2 above control
Untreated	K562 cells	68 ± 28
PTX treated (2·5 μg/ml)	K562 cells	91 ± 8

Experiment 2

Treatment	Stimulus	% increase in 1,4,5 IP_3 above control
Untreated	K562 cells	23 ± 13
PTX treated (2·5 μg/ml)	K562 cells	24 ± 6

Experiment 3

Treatment	Stimulus	% increase in 1,4,5 IP_3 above control
Untreated	K562 cells	69 ± 1
PTX treated (2·5 μg/ml)	K562 cells	77 ± 36

an elevation in cAMP levels. However, treatment of NK cells with 2·5 μg/ml PTX produced no change in the intracellular cAMP level (Table 1).

These results indicate that an elevation in intracellular cAMP, while known to inhibit NK cytotoxicity, is unlikely to be responsible for the inhibition seen after PTX treatment.

PTX treatment of NK-cell membranes ADP ribosylates an approximately 41,600 M_r substrate

Figure 2a shows an autoradiogram of a SDS-PAGE [10% (w/v) polyacrylamide gel] of NK-cell membranes which were incubated with [^{32}P]NAD^+, with and without PTX. The PTX-treated membranes showed a labelled band at a M_r of approximately 41,600 which corresponds roughly to the M_r of PTX-sensitive Gαs in other cell types, these include G_i and G_o.[5] When membranes from cells that were pretreated with PTX (2·5 μg/ml) were incubated with [^{32}P]NAD^+ in the presence of PTX the labelling of the 41,600 M_r band was diminished (Fig. 2b).

Effect of PTX on effector–target cell binding

The ability of NK cells to bind to the K562 target cells was compared between PTX-treated and control cells (Fig. 3). PTX treatment had no significant effect on the fraction of target cells with NK cells bound to them at any of the effector cell/target cell ratios examined. These results indicate that binding of NK cells to the target cells is not being significantly inhibited due to PTX treatment.

Effect of PTX 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 cytotoxic factors from NK cells.[18] Table 2 shows that PTX treatment does not cause significant granule release from NK cells as compared to the release stimulated by PMA and A23187. Thus release of cytotoxic factors during preincubations of NK cells with PTX is not likely to be responsible for the decrease in NK cytotoxicity. Table 2 also shows that control cells and those that were pretreated with PTX released the same percentage of their total β-glucuronidase when stimulated by PMA and A23187, indicating that releasable-granule number is not affected by PTX treatment.

Effect of PTX treatment on NK-cell locomotion

NK cells move toward the chemoattractants IL-2 and FMLP (Table 3). In order to see if NK locomotion was in any way affected by PTX treatment, we compared the movement of PTX and control cells toward these two chemoattractants. Table 3 shows that pretreatment with 2·5 μg/ml PTX inhibits movement of NK cells toward IL-2 by approximately 50% and completely blocks their movement toward FMLP.

Effect of PTX treatment on NK-cell phospholipase C activity

Binding to target cells is known to stimulate NK-cell PLC activity and phosphoinositide turnover.[4] We measured NK PLC activity to look for PTX-sensitive G protein involvement in PLC activation. Stimulation of PLC activity by target cells was measured and there was an increase in inositol 1,4-bisphosphate (1,4 IP_2) or inositol 1,4,5-trisphosphate (1,4,5-IP_3) turnover when NK cells were exposed to K562 cells (Table 4). PTX pretreatment of NK cells did not affect the target cell stimulation of PLC.

DISCUSSION

The data presented indicate that NK-cell cytotoxicity is inhibited by PTX treatment. We investigated the effects of PTX treatment on several NK-cell functions (cAMP levels, binding to target cells, release of cytotoxic granules, PLC activation by target cells and chemotaxis) in order to determine which step(s) in the cytotoxic process are sensitive to PTX treatment. One possible explanation for inhibition of any step in the cytotoxic process by PTX treatment is that it might involve a PTX-sensitive G protein(s).

There is a substrate for PTX-specific ADP ribosylation with M_r 41,600 in NK cells. This M_r is very similar to that of PTX sensitive Gαs. The fact that treatment of NK cells with the B subunit of PTX, which cannot ADP ribosylate Gα,[19] does not inhibit NK cytotoxicity is also suggestive of a PTX-sensitive Gα being involved in the cytotoxic process.

The results suggest that PTX is not inhibiting NK cytotoxicity by inactivating Gi for adenylate cyclase since no elevation of cAMP could be measured. Had PTX elevated NK-cell cAMP this could have explained the inhibition of cytotoxicity, as elevated cAMP is known to inhibit NK-cell cytotoxicity.[4,6,20–23] Furthermore, decreased binding of NK cells to target cells, or effects on cytotoxic-granule release do not appear to be involved in the inhibition of cytotoxicity by PTX. It is possible that a PTX-sensitive G protein is linked to activation of PLC by target cells. However, PTX treatment had no effect on PLC activation by target cells indicating that if there is a G protein linked to this PLC it is not PTX sensitive.

The fact that PTX treatment is able to inhibit completely the movement of NK cell towards the chemoattractant FMLP suggests that directed motion towards FMLP may involve a PTX-sensitive G protein. A PTX-sensitive G protein is known to be linked to FMLP receptors in neutrophils and is involved in the activation of PLC by FMLP.[24] The PTX substrate for ADP ribosylation in neutrophils has a M_r similar to one that we have found in NK cells.[24,25] We are now investigating the involvement of a PTX-sensitive G protein in activation of NK-cell PLC by FMLP.

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