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CYCLIC GUANOSINE 3′,5′-MONOPHOSPHATE MEDIATES 3-MORPHOLINOSYDNONIMINE-INDUCED INHIBITION OF HUMAN NATURAL KILLER CELLS

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Abstract—Nitric oxide (NO) donors were used to investigate the effect of NO on and the role of cyclic GMP in the regulation of human natural killer (NK) cell function. NO-producing drugs, molsidomine and its metabolite 3-morpholinesydnonimine (SIN-1), inhibited NK cell-mediated cytotoxicity significantly at 0.04–5 mM. At 1 mM, SIN-1 completely inhibited NK cell activity while molsidomine decreased NK cell-mediated cytolysis by 35% of the control value. These data suggest that NO from exogenous NO-donors may down-regulate NK cell cytotoxic function. The stimulatory effect of interferon-γ (IFN-γ) on human NK cell-mediated killing could not overtake the NK cell inhibition induced by the NO releasing drugs, indicating different modes of action for IFN-γ and SIN-1. The results in the present study also showed that SIN-1 (1 mM) stimulated cyclic GMP production 37-fold in NK cells. In the presence of 0.5 mM IBMX, a phosphodiesterase inhibitor, the increase in cyclic GMP was even more pronounced, demonstrating a relation between cyclic GMP stimulation and NK cell inhibition by SIN-1. Further evidence for mediation via cyclic GMP was provided by the finding that methylene blue (20 μM), an inhibitor of soluble guanylate cyclase, decreased both the inhibition of SIN-1-induced NK cell cytotoxicity as well as cyclic GMP formation. Moreover, membrane-penetrating cyclic GMP and its analogues inhibited NK cell-mediated cytolysis significantly. Molsidomine was without effect on cyclic GMP levels. Our data indicate that cyclic GMP may play a role in human NK cell regulation and suggest that the inhibitory effect of cGMP may be elicited by NO.

Key words: cGMP; nitrovasodilators; nitric oxide; NK cell

NO† is an intermediate metabolite of L-arginine [1] and has multiple biologic functions. It has been shown that the endothelium-derived relaxing factor, which mediates vasodilation and inhibition of platelet aggregation [2], is NO [3]. In addition, NO has multiple messenger functions in the brain [4, 5]. In particular, NO has been demonstrated to be an important signal substance in the regulation of cell functions and cell–cell communication in the immune system, including the inhibition of leukocyte adhesion [6], the regulation of polymorphonuclear leukocytes [7], and the antimicrobial or anti-tumour effects mediated by macrophages [8, 9]. One study has indicated that NO is also involved in the regulation of T-lymphocyte proliferation [10]. Macrophages inhibit T-cell responses to mitogens by production of NO from L-arginine [11, 12]. NK cells are the subpopulation of lymphocytes with a direct spontaneous cytotoxicity against tumour cells, virus-infected cells and some haematopoietic cells of normal origin. A large number of studies have suggested that NK cell cytotoxicity may form an important part of the non-specific host defence against tumour cells and microorganisms [13]. To date, little is known about the influence of NO on NK cell function. Since NO or NO generators are known to be potent activators of soluble guanylate cyclase [14, 15], they may be considered as suitable tools to assess the role of intracellular cyclic GMP in the regulation of cellular activity. The purpose of the present study was to investigate the effect of the NO generator SIN-1 and its precursor, MOL, on NK cell-mediated cytolysis of a tumour cell line *in vitro* and to determine whether NO and cyclic GMP interact in governing NK cell activity *in vitro*.

MATERIALS AND METHODS

Materials. Ficoll-Paque and Percoll were from Pharmacia (Uppsala, Sweden). Cyclic GMP, 8-br-cGMP, db-cGMP, methylene blue and IBMX were purchased from Sigma (St. Louis, MO, U.S.A.). SIN-1 and MOL were gifts from Hoechst AB (Stockholm, Sweden).

Preparation of NK cell-enriched effector cells and target cells. PBL were isolated from heparinized venous blood from healthy adult volunteers 25–35 years of age by Ficoll-Paque density gradient centrifugation. To deplete adherent monocytes [16], PBL were incubated in plastic tissue culture flasks in 5% CO_2 for 1 hr at a concentration of 1×10^6 cells/mL CM consisting of RPMI 1640 medium, 10% foetal calf serum, 100 U penicillin/mL and 100 μg streptomycin/mL. To achieve maximal purity, the

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† Abbreviations: NO, nitric oxide; NK, natural killer; SIN-1, 3-morpholinosydnonimine; MOL, molsidomine; 8-br-cGMP, 8-bromo cyclic GMP; db-cGMP, dibutyryl cyclic GMP; IBMX, isobutylmethylxanthine; PBL, peripheral blood lymphocyte; CM, complete medium; EC, effector cell; TC, target cell; HBSS, Hank's buffered saline solution; IFN-γ, interferon-γ.

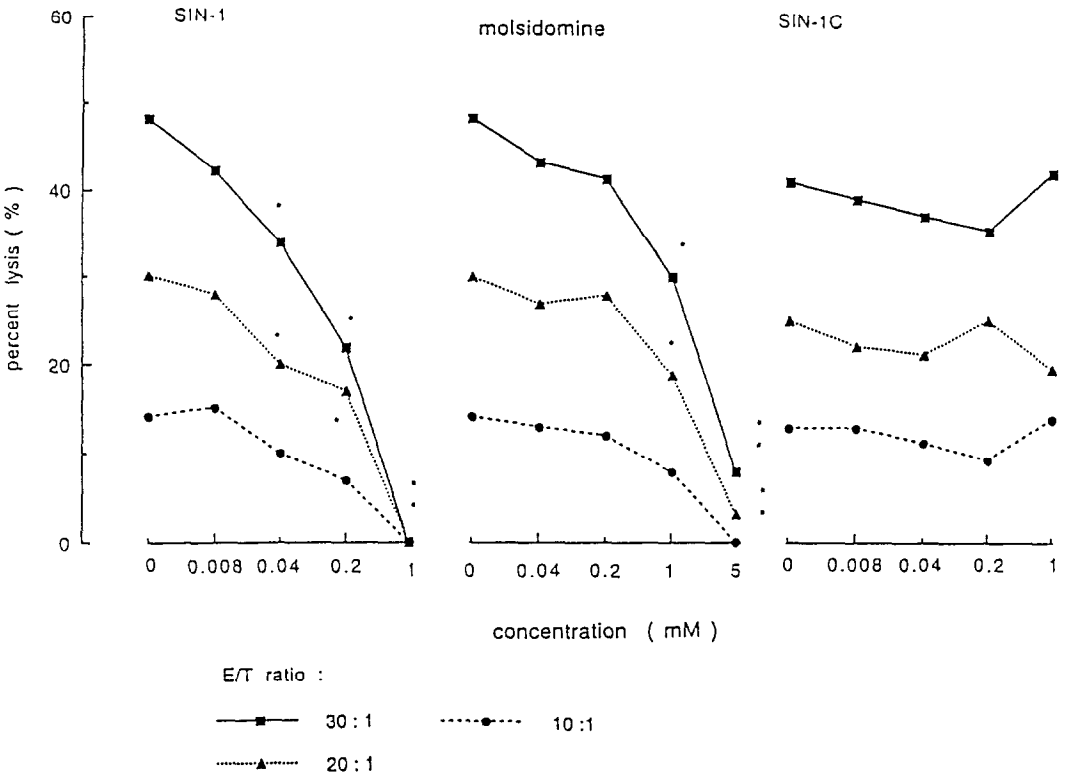

Fig. 1. Dose-dependent inhibition effect of SIN-1 and MOL on NK cell activity. SIN-1, SIN-1C or MOL was present throughout the 4 hr cytotoxicity assay at the indicated concentration with different EC/TC ratios. Each point represents the mean of five experiments using different blood donors. SEM was less than 6% at any concentration of SIN-1 and MOL and error bars have been omitted for the sake of clarity. $^{*}P < 0.05$, $^{**}P < 0.01$ (treatment versus control).

non-adherent cells were subjected a second time to the same adherence protocol as described above, the only difference being that the adherence time was 30 min. The non-adherent cells recovered from the second adherence contained less than 1% of monocytes (as determined by the non-specific esterase stain) and were applied to further Percoll-isolation to give NK cell-enriched mononuclear cells. The method used was similar to that described by Timonen and Saksela [17]. Briefly, 9 volumes of Percoll was made iso-osmolar by addition of 1 volume of 1.5 M saline. Osmolality was adjusted to 290 mosmol/kg. Discontinuous density gradients were prepared by mixing Percoll at various concentrations with CM. The gradient was layered in a 50 mL plastic tube with 50% Percoll at the bottom, finally arriving after 2.5% diminutions to 37.5% Percoll mixed with approximately 6×10^7 cells at the top. The tube was centrifuged at 280 *g* for 40 min at room temperature. Five millilitre portions of the gradient were then collected from the top yielding a total of six fractions, the first three of which were enriched in large granular lymphocytes accounting for human NK cell activity [17]. These three fractions were employed as effector cells in all experiments.

The human erythroleukaemic cell line K_{562} was used as target cell in the NK cell cytolysis assay. Approximately 3×10^6 TC, suspended in 0.3 mL of CM, were incubated for 60 min at 37° in the presence of 150 μCi of ^{51}Cr sodium chromate (Amersham, U.K.). After centrifugation and resuspension in CM, 10^4 cells in 10 μL portions were added to the effector cells in the microplate wells.

NK cell-mediated cytolysis assay. EC : TC ratios of 30 : 1, 20 : 1, 10 : 1 were used. The EC/TC suspensions were incubated in triplicate wells of microtiter plates for 4 hr at 37°. Thereafter, supernatant fluids were collected and assayed for radioactivity in a γ-counter (LKB). NK cell activity was calculated according to the following formula: percent lysis (%) = (experimental ^{51}Cr-release − spontaneous ^{51}Cr-release)/(maximal ^{51}Cr-release − spontaneous ^{51}Cr-release). Maximum ^{51}Cr-release was determined in cultures treated with 6 N HCl. Substances to be tested were dissolved in CM and added to the mixture of EC and TC during the assay.

Target binding cell assay. EC-TC conjugates were enumerated as described previously [18]. In brief, 1×10^6 EC and 1×10^6 unlabelled TC were mixed in 0.5 mL of CM in a polypropylene tube and centrifugated at 40 *g* for 5 min. The cell mixture was incubated at 37° for 15 min. Thereafter, the cell pellet was carefully resuspended. A small aliquot was removed and placed into a haemocytometer. Enumeration was accomplished by quadriplicate

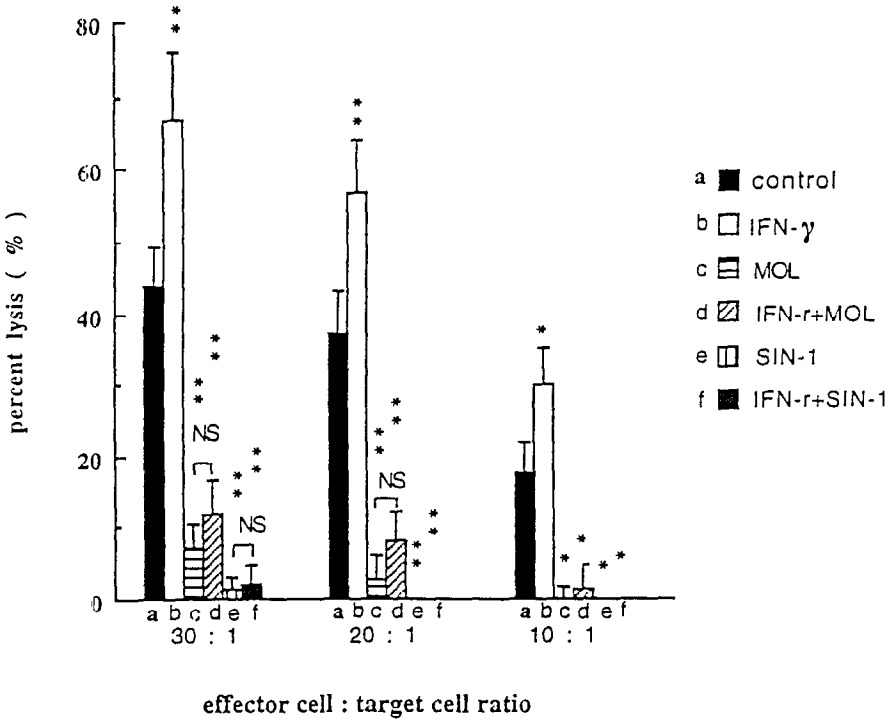

Fig. 2. Effect of SIN-1 and MOL on IFN-γ treated effector cells. Effector cells were preincubated with IFN-γ (200 U/mL) for 18 hr and then tested for NK activity in the presence of SIN-1 (1 mM) or MOL (5 mM). Results represent the mean ± SEM of five experiments using different blood donors. *P < 0.05, **P < 0.01 (treatment versus control).

counting of the number of conjugates per 200 lymphocytes.

Cyclic GMP and cyclic AMP assay. EC were incubated in 0.5 mL of HBSS with various compounds for 1 hr at 37° unless otherwise indicated. The EC suspension was disrupted by sonication at 4°. Ice-cold ethanol was added to the cell suspension to give a final suspension volume of 65% ethanol. The resulting homogenate was centrifugated at 2000 *g* for 15 min at 4° and the supernatant fraction collected. Cyclic GMP and cyclic AMP were measured by radioimmunoassay as described previously [19, 20] using the nucleotide assay kits from Amersham (U.K.).

Statistical analysis. The results are expressed as the mean ± SEM for N different blood donors. Differences were analysed by Student's two-tailed *t*-test. P values of < 0.05 were considered significant.

RESULTS

Effect of SIN-1 and MOL on human NK cell activity

Addition of MOL and SIN-1 in concentrations of 0.04–5 mM to the mixture of EC and TC with different EC:TC ratios at the onset of the 4 hr ^{51}Cr-release assay resulted in significant and dose-dependent inhibition of NK cell-mediated cytotoxicity (Fig. 1). SIN-1C, an inactive decomposition product of SIN-1, had no effect on NK cell activity. At 1 mM, SIN-1 completely inhibited NK cell activity while MOL inhibited NK cell activity by 35% of the control value. ED_{50} was 0.12 mM for SIN-1 and 2.1 mM for MOL. Data in Fig. 2 show that treatment with IFN-γ failed to overcome the inhibitory effects of SIN-1 and molsidomine. IFN-γ (200 U/mL) alone increased NK activity by 50%. SIN-1 and MOL inhibited NK cell cytolysis to the same degree irrespective of IFN-γ treatment. Further experiments indicated that SIN-1 and MOL acted at the level of the NK effector cell. NK effector or ^{51}Cr-labelled target cells were pretreated with SIN-1, MOL or HBSS (control) and were then washed and admixed in a 4 hr cytolysis assay. Pretreatment with SIN-1 and MOL did not induce target cell resistance to NK cell-mediated lysis (Fig. 3).

Effect of MOL and SIN-1 on frequency of target binding cells

Data in Table 1 show that neither MOL nor SIN-1 affected binding between EC and TC.

Effect of MOL and SIN-1 on intracellular cyclic GMP and cAMP

The basal cyclic GMP level was 6 ± 0.5 fmol/6×10^5 cells (N = 7) and was the same in both HBSS and CM (data not shown). SIN-1 significantly increased the intracellular cyclic GMP level. At 0.01 mM SIN-1 produced a 9-fold or, in the presence of IBMX, a 27-fold increase in cyclic GMP

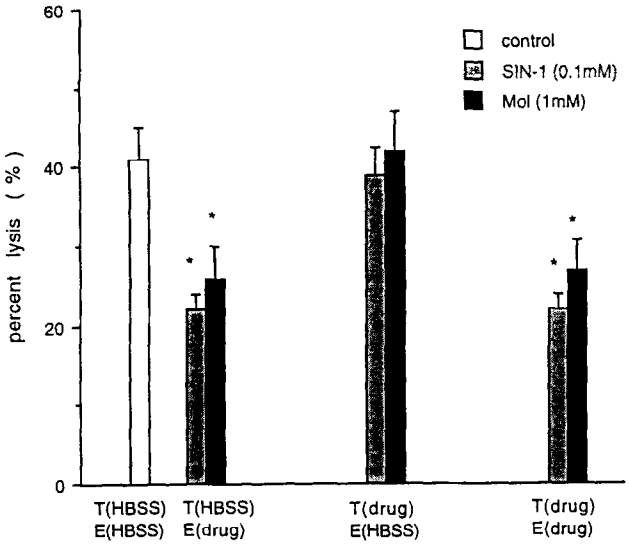

Fig. 3. SIN-1 and MOL act at the level of the NK effector cell. NK effector cells or ^{51}Cr-labelled targets at the same concentration were pretreated for 30 min at 37° with SIN-1, MOL or HBSS (control), respectively, and then washed three times with CM and admixed at a EC/TC ratio of 30:1 in a 4 hr cytolysis assay. *$P < 0.05$ (treatment versus control).

Table 1. Effect of MOL and SIN-1 on frequency of target binding cells (TBC)

Treatment	TBC (%)*
Control	14.8 ± 2.4
MOL (1 mM)	13.4 ± 3.7
SIN-1 (0.2 mM)	12.7 ± 3.9

* As determined by a liquid single cell assay in the presence of MOL or SIN-1. Data are mean ± SEM of quadruplicate determinations with monocyte-depleted mononuclear cells from each of three donors.

(Fig. 4). With 1 mM of SIN-1, cyclic GMP stimulation increased to 37-fold or, in the presence of IBMX, 83-fold. There was no significant change in cyclic GMP with MOL (Fig. 4). However, MOL (5 mM) in the presence of IBMX produced a significant increase in cyclic GMP to 14-fold of control level. The cyclic AMP level was not significantly affected by SIN-1 nor MOL in the presence or absence of IBMX (data not shown).

Effect of IBMX on the time course of cyclic GMP accumulation

The time course of the effect of 0.5 mM IBMX on cyclic GMP accumulation in NK cells in the presence of 0.1 mM of SIN-1 is shown in Fig. 5. In the presence of IBMX, SIN-1 rapidly and continuously produced an increase in cyclic GMP with maximal cyclic GMP accumulation occurring at 30 min (40-fold increase). After 30 min the cyclic GMP level remained relatively constant but there was a significant difference between the SIN-1 and the SIN-1+IBMX experiments. IBMX appeared to potentiate the SIN-1 induced accumulation of cyclic GMP. The basal cyclic GMP level was not significantly elevated by IBMX alone.

Effect of cyclic GMP and its analogues on NK cell activity

Adding 0.1 mM or 1 mM of cyclic GMP to the mixture of EC and TC during a 4 hr cytotoxicity assay significantly inhibited NK cell-mediated cytolysis by 22% or 37% as compared to the control experiments (N = 4) (Fig. 6). 8-br-cGMP (5 mM) and db-cGMP (5 mM) produced a significant reduction in NK cell activity of 35% or 25% that observed in control experiments. There were no significant differences between the effects of cGMP and its analogues except that obtained at the highest concentration (5 mM). Similarly, pretreatment of target cells alone with cyclic GMP or its analogues did not cause a significant alteration of NK activity against target cells (data not shown).

Effect of methylene blue on NK cell inhibition by SIN-1 and MOL

The guanylate cyclase inhibitor, methylene blue, attenuated the inhibitory effect of SIN-1 on NK cell activity as well as the SIN-1-induced increase in intracellular cyclic GMP. In the presence of methylene blue (20 μM), NK cell activity inhibition by SIN-1 (0.1 mM) was decreased from 54% to 30% of the control value. Cyclic GMP stimulation by SIN-1 was reduced at the same time. However, methylene blue had no effect on the MOL-induced inhibition of NK cell activity. Cyclic GMP levels were not significantly increased by MOL and also remained unaltered in the presence of methylene blue (Fig. 7).

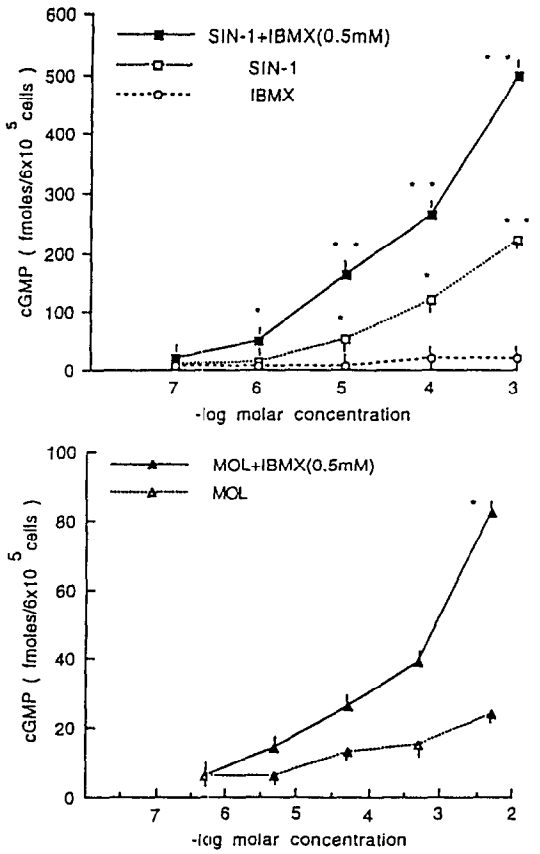

Fig. 4. Effect of SIN-1 and MOL on cyclic GMP levels in NK effector cells. Effector cells were incubated with SIN-1 or MOL at the indicated concentration for 1 hr in the presence or absence of the phosphodiesterase inhibitor IBMX (0.5 mM) and then tested for cyclic GMP. Results represent the mean ± SEM of four experiments using different blood donors. *P < 0.05, **P < 0.01 (treatment versus control). The average cyclic GMP level in control cells was 6 ± 0.5 fmols/6 × 10^5 cells (N = 7).

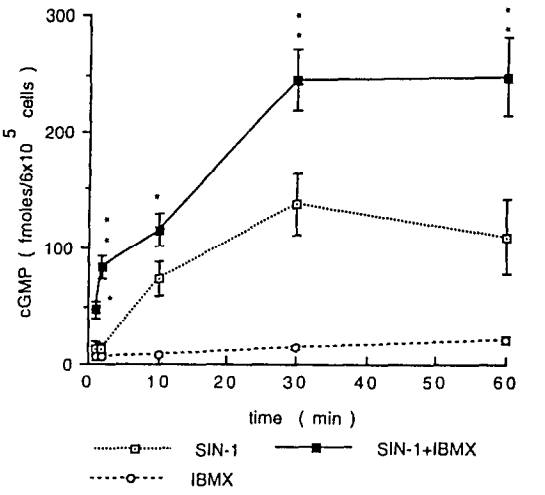

Fig. 5. Effect of IBMX on the time course of cyclic GMP accumulation in NK effector cells exposed to SIN-1. Effector cells were incubated with SIN-1 (0.1 mM) and/or IBMX (0.5 mM) for various time periods and then tested for cyclic GMP (N = 4 for each experiment). IBMX had no significant effect on the basal cGMP level. *P < 0.05, **P < 0.01 (SIN-1+IBMX versus SIN-1).

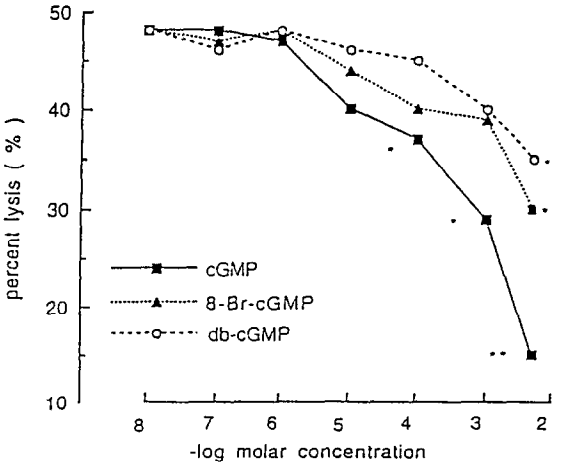

Fig. 6. Effect of cyclic GMP and its analogues, db-cGMP and 8-br-cGMP, on NK cell activity (N = 4 for each point). E/T ratio was 30:1. SEM were less than 7% in all experiments and error bars were omitted for the sake of clarity. The control percent lysis was 48 ± 4%. *P < 0.05, **P < 0.01 (treatment versus control).

DISCUSSION

Since they are potential NO generators [15] MOL and its metabolite SIN-1 were used to investigate the biological role of NO. The data in the present study indicate that SIN-1 as well as MOL cause a substantial inhibition of human NK cell-mediated cytotoxicity *in vitro*. SIN-1C, an inactive decomposition product of SIN-1, had no effect on NK cell activity. The inhibition of NK cell activity by SIN-1 or MOL seems not to be due to toxic effects of these drugs as the incubation of NK effector cells with SIN-1 or MOL even in as high a concentration as 10 mM did not affect cell viability (data not shown). SIN-1 and MOL did not decrease effector–target cell conjugate formation nor did they influence target cell resistance to NK lysis. Thus SIN-1 and MOL act at the level of the NK effector cell and may interfere with NK cell-induced target cell lysis by affecting the killing mechanism after binding. This result opens the possibility that NO generated *in vivo* by endothelial cells [21] as well as leukocytes and macrophages [8, 9] may take part in the regulation of NK cells. The participation of NO in the control of cell functions has been demonstrated. NO may thus inhibit human polymorphonuclear leukocytes [7], platelet aggregation [22], smooth muscle cells [23] and macrophages [24].

NO is naturally formed by conversion of L-arginine

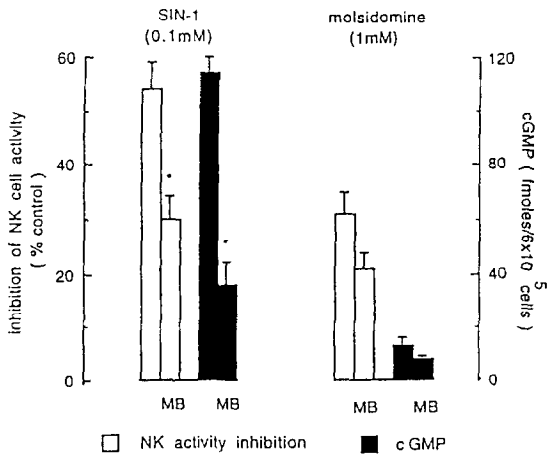

Fig. 7. Effect of MB on inhibition of NK cell-mediated cytolysis of target cells (open columns) and cyclic GMP levels (filled columns) in the presence of SIN-1 or molsidomine (N = 4 for each column). EC/TC ratio was 30:1. *P < 0.05 (MB versus no MB).

to citrulline and NO [1]. Recently, it was found that per oral administration of L-arginine led to an activation of human NK cells [25]. This stimulatory effect of L-arginine was found to be mediated by the NO synthase pathway [26]. The difference between these *in vivo* findings and our *in vitro* results suggests that the generation of NO from exogenous precursors may be operative in a different fashion from that observed during endogenous NO release. As SIN-1 may give rise to NO spontaneously whereas arginine is converted by constitutive or inducible enzymes [1, 27] the generation of endogenous, not exogenous, NO may be compartmentalized. NO derived from L-arginine metabolism may thus be mainly distributed to target cells and not influence NK cells themselves. In contrast, exogenous NO produced from NO-donors could presumably react with NK effectors. Furthermore, it is not entirely sure that the same type of NO is formed from NO-donors (i.e. SIN-1) and arginine, possibly leading to different interactions of NO with superoxide anion and thiols [28]. NO generation may cause cytotoxic effects. It is thus likely that exogenous NO could affect NK cell function such as mitochondrial activity [9] or inhibit the NO synthase effector pathway as demonstrated previously [29] without participating in the killing mechanism itself. A biphasic effect of NO donors has been demonstrated in polymorphonuclear leukocytes [30]. However, in our experiments SIN-1 was found only to inhibit—never stimulate—human NK cells over a wide concentration range. Interestingly, similar results regarding the different effects of endogenously and exogenously formed NO on macrophages [8, 9, 24] and leukocytes [7, 30, 31] have also been reported.

Because IFN-γ may stimulate NK effector cells, we examined if this biologic response modifier could overcome the inhibition of NK cell cytotoxicity by SIN-1 or MOL. The results show that SIN-1 and MOL inhibited NK cell-mediated cytolysis of target cells despite the presence of stimulatory amounts of IFN-γ, indicating that the drugs and IFN-γ may operate in different ways. The mode of action of IFN-γ on human NK cells is unknown. It has, however, been reported that IFN-γ in synergy with tumour necrosis factor could induce NO production accompanied by an inhibition of smooth muscle cells [23, 32]. If IFN-γ stimulates NO formation in human NK cells this should have occurred via NO synthase and arginine, whereas NO releasing drugs do not seem to engage such a pathway. Endogenously-released NO appears to participate in the killing mechanism whereas exogenously released NO seems to interfere with the regulation of NK cell function. It may be speculated that the latter process is more important in target cell killing.

It is well known that NO may bind to a heme moiety in the structure of the soluble form of guanylate cyclase, resulting in guanylate cyclase activation and cyclic GMP generation. Cyclic GMP has been demonstrated to be a messenger mediating multiple biologic effects of NO [7, 14, 33]. To date, little is known about the role of cyclic nucleotides in the regulation of NK cell function. In the present study we found that the inhibitory effect of SIN-1 on NK cell cytotoxicity was associated with a concomitant and sustained increase in intracellular cyclic GMP. In the presence of IBMX, the increase in cyclic GMP was even more pronounced and stable. These results indicate that NK cells have the soluble form of guanylate cyclase which can be stimulated by NO. Further evidence for mediation via cyclic GMP was provided by the finding that methylene blue decreased both SIN-1 induced NK cell cytotoxicity inhibition and cyclic GMP stimulation. Methylene blue at the concentration used is known to be a selective inhibitor of the soluble isoenzyme of guanylate cyclase [34]. The inhibitory function of cyclic GMP during NK cell activation is further substantiated by the finding that membrane-penetrating cyclic GMP and its analogues also decrease NK activity.

In contrast to SIN-1, molsidomine produced a less marked yet significant decrease in NK cell cytotoxic function. This inhibitory effect was not accompanied by a significant increase in intracellular cyclic GMP and was not attenuated by methylene blue. Even though the combined presence of molsidomine and IBMX yielded a significant increase in NK cell contents of cyclic GMP, it seems possible that MOL in NK cells was either converted to SIN-1 to some degree or acted on some other cell function with only a limited effect on cyclic GMP. In human polymorphonuclear leukocytes and platelets, similar cyclic GMP-independent inhibitory effects of MOL were reported [7, 35] suggesting a non-specific, direct effect of MOL. Molsidomine is a mesoionic compound with a permanent negative and positive charge. It is thus likely that by exerting electrostatic effects, MOL is capable of stabilizing target cell membrane and lysosomal membranes of effector cells leading to the inhibition of NK cell cytotoxic ability.

In summary, we have demonstrated for the first time that NO may down-regulate human NK cell-

mediated cytolysis of the tumour cell line *in vitro* and that this inhibition could not be overcome by IFN-r. This inhibitory effect of NO was associated with a concomitant and sustained increase in intracellular cyclic GMP, indicating that cyclic GMP as a messenger may play a role in the regulation of human NK cell function.

REFERENCES

1. Marietta MA, Poksyn SY, Lyengar R and Leaf CD, Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry* **27**: 8706, 1988.
2. Ignarro LJ, Nitric oxide: a novel signal transduction mechanism for transcellular communication. *Hypertension* **16**: 477, 1990.
3. Palmer RMJ, Ferrige AG and Moncada S, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**: 524, 1987.
4. Bredt DS, Hwang PM and Snyder SH, Localization of nitric oxide synthase indicates a neural role for nitric oxide. *Nature* **347**: 768, 1990.
5. Shibuki K and Okada D, Endogenous nitric oxide release is required for long-term synaptic depression in the cerebellum. *Nature* **349**: 326, 1991.
6. Kubes P, Suzuki M and Grager DN, Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci USA* **88**: 4651, 1991.
7. Schröder H, Ney P, Woditsch I and Schrör KF, Cyclic GMP mediates SIN-1-induced inhibition of human polymorphonuclear leukocytes. *Eur J Pharmacol* **182**: 211, 1990.
8. Leiw FY and Cox FEG, Nonspecific defence mechanisms: the role of nitric oxide. *Immunol Today* **12**: 417, 1991.
9. Hibbs JB, Taintor RR, Vavrin Z and Rachlin EM, Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* **157**: 87, 1988.
10. Hoffman RA, Langehr JM, Billiar TR, Curran RD and Simmons RL, Alloantigen induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *J Immunol* **145**: 2220, 1990.
11. Mills CD, Molecular basis of "suppressor" macrophages: arginine metabolism via the nitric oxide synthesis pathway. *J Immunol* **146**: 2719, 1991.
12. Albina JE, Abate JA and Henry WL Jr, Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation: role of IFN-r in the induction of the nitric oxide-synthesizing pathway. *J Immunol* **147**: 144, 1991.
13. Lewis CE and McGee JO'D, *Natural Killer Cell*. Oxford University Press, New York, 1992.
14. Moncada S, Palmer RMJ and Higgs EA, Nitric oxide: physiology, pathology, and pharmacology. *Pharmacol Rev* **43**: 109, 1991.
15. Schrör KF, Woditsch I and Schröder H, Generation of NO from molsidomine (SIN-1) in vitro and its relationship for changes in coronary vessel tone. *J Cardiovasc Pharmacol* **14** (supplement 11): S4, 1989.
16. Fischer DG, Hubbard WJ and Koren HS, Tumor cell killing by freshly isolated peripheral blood monocytes. *Cell Immunol* **58**: 426, 1981.
17. Timonen T and Saksela E, Isolation of human NK cell by density gradient centrifugation. *J Immunol Meth* **36**: 285, 1980.
18. Abrams SI, Bray RA and Brahmi Z, Mechanism of action of phorbol myristate acetate on human natural killer cell activity. *Cell Immunol* **80**: 230, 1983.
19. Steiner AL, Parker CW and Kipnis DM, Radioimmunoassay of cyclic nucleotides. *J Biochem* **247**: 1106, 1972.
20. Harper JF and Brooker G, Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'0 acetylation by acetic anhydride. *J Cycl Nucl Res* **1**: 207, 1975.
21. Ignarro LJ, Buga GM, Wood KS, Byrns RE and Haudhuri G, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* **84**: 9265, 1987.
22. Marletta MA, Tayeh MA and Hevel JM, Unraveling the biological significance of nitric oxide. *Bio Factors* **2**: 219, 1990.
23. Geng Y-J, Hansson GK and Holme E, Interferon-γ and tumor necrosis factor synergize to induce NO production and inhibit mitochondrial respiration in vascular smooth muscle cells. *Circ Res* **71**(5): 1268, 1992.
24. Drapier JC and Hibbs JB Jr, Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. *J Immunol* **140**: 2829, 1988.
25. Park KGM, Hayes PD, Garlick PJ, Sewell H and Eremin O, Stimulation of lymphocyte natural cytotoxicity by L-arginine. *Lancet* **337**: 645, 1991.
26. Cifone MG, Festuccia C, Cironi L, Cavallo G, Chessa MA, Pensa V, Tubaro E and Santon A, Induction of the nitric oxide-synthesizing pathway in fresh and interleukin 2-cultured rat natural killer cells. *Cell Immunol* **157**: 181, 1994.
27. Bohn H and Schönafinger K, Oxygen and oxidation promote the release of nitric oxide from sydnonimines. *J Cardiovasc Pharmacol* **14** (supplement 11): S6, 1989.
28. Lipton SA, Chol YB, Pan H, Lel SZ, Chen H-SV, Sucher NJ, Loscalzo J, Singel DJ and Stamler JS, A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364**: 626, 1993.
29. Griscavage JM, Rogers NE, Sherman MP and Ignarro LJ, Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. *J Immunol* **151**: 6329, 1993.
30. Pieper GM, Clarke GA and Gross GJ, Stimulatory and inhibitory action of nitric oxide donor agents vs. nitrovasodilators on reactive oxygen production by isolated polymorphonuclear leukocytes. *J Pharmacol Exper Ther* **269**: 451, 1994.
31. Salvemini D, De Nucci G, Gryglewski RJ and Vane JR, Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci USA* **86**: 6328, 1989.
32. Nunokawa Y and Tanaka S, Interferon-γ inhibits proliferation of rat vascular smooth muscle cells by nitric oxide generation. *Biochem Biophys Res Commun* **188**: 409, 1992.
33. Ohigahi T, Brookins J and Fisher JW, Interaction of nitric oxide and cyclic guanosine 3',5'-monophosphate in erythropoietin production. *J Clin Invest* **92**: 1587, 1993.
34. Leitman DC, Agnost VL, Catalano RM, Schröder H, Waldman SA, Bennett BM, Tuan JJ and Murad F, Atrial natriuretic peptide, oxytocin, and vasopressin increase guanosine 3',5'-monophosphate in LLC-PKT kidney epithelial cells. *Endocrinology* **122**: 1478, 1988.
35. Nishikawa M, Kanamori M and Hidaka H, Inhibition of platelet aggregation and stimulation of guanylate cyclase by an antianginal agent molsidomine and its metabolites. *J Pharmacol Exper Ther* **220**: 183, 1982.