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Inhibition of natural killer (NK) cell activity against varicella-zoster virus (VZV)-infected fibroblasts and lymphocyte activation in response to VZV antigen by nitric oxide-releasing agents

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SUMMARY

The addition of nitric oxide (NO)-releasing agents, S-nitroso-N-acetyl-DL-penicillamine (SNAP), 1-hydroxy-2-oxo-2,3-bis(2-aminoethyl)-1-triazene (NOC18), 30{(±)-(E)-ethyl-2′-[(E)-hydroxyimino]-5-nitro-3-hexenecarbamoyl}-pyridine (NOR4) significantly inhibited NK cell activity against VZV-infected cells, while antibody-dependent cell-mediated cytotoxicity (ADCC) against VZV-infected cells was unaffected. Interferon-alpha (IFN-α) production by non-adherent peripheral blood mononuclear cells (NPBMC) cultured with VZV-infected cells was decreased by the addition of NO-releasing agents. Lymphocyte proliferation and the expression IL-2 receptor (CD25) in response to VZV antigen were also inhibited by the addition of NO-releasing agents. These results suggest that the production of NO by an inflammatory process may lead to inhibition of NK cell- and T cell-mediated immunity to VZV infection.

Keywords varicella zoster virus natural killer cell nitric oxide

INTRODUCTION

Nitric oxide (NO) has multiple biological functions, including vascular relaxation [1], and tumourcidal and antimicrobial activities [2,3]. Inflammatory cytokines, including interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and IL-1, can induce production of NO by endothelial cells and neutrophils [4,5]. Excessive production of NO by inflammatory cytokines may influence the host immune response. It is important to investigate the effects of NO on host defence mechanisms.

VZV, a human herpes virus, causes varicella and shingles. Peripheral blood mononuclear cells (PBMC) mediate NK and antibody-dependent cell mediated-cytotoxicity (ADCC) against VZV-infected fibroblasts [6]. PBMC from healthy persons with a history of varicella proliferate and the expression of IL-2 receptor on T cells increases in response to VZV antigen in culture [7]. Both killing activity mediated by NK cells and activation of T cells are important in recovering from VZV infection. In the present study, we investigated the effects of NO-releasing agents on NK and ADCC activity against VZV-infected cells and lymphocyte proliferation in response to VZV antigen.

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MATERIALS AND METHODS

Mononuclear cell preparations

PBMC were separated from heparinized venous blood of VZV^+ healthy adults by Ficoll–Hypaque (Sigma, St Louis, MO) gradient centrifugation. PBMC at the interface were collected and washed three times with RPMI 1640 (GIBCO BRL, Grand Island, NY), then suspended in RPMI 1640 with 20% autologous serum at a concentration of 1×10^6/ml and used for proliferation assay. As effector cells for NK and ADCC assay, non-adherent PBMC (NPBMC) were prepared by incubating PBMC suspended in RPMI 1640 containing 10% fetal bovine serum (FBS; GIBCO BRL) in plastic Petri dishes coated with FBS, as described previously [8].

Preparation of target cells

Human foreskin fibroblasts (FS-4) were cultured in minimum essential medium (MEM) (GIBCO BRL), supplemented with 10% FBS. The FS-4 cells were used between passages 13 and 19. Confluent FS-4 cells were infected with cell-free VZV (Kawaguchi strain) at a multiplicity of infection of 0·01. When 80–90% of the cells showed cytopathic effect (CPE), cells were removed with 0·25% trypsin–1 mM EDTA 4 Na (GIBCO BRL) and added to uninfected FS-4 cells at a ratio of one flask of infected cells to five flasks of uninfected cells. After 48 h of incubation at 37°C, the infected cells were removed with 0·25% trypsin–1 mM EDTA 4 Na and suspended in MEM with 10% FBS–10% dimethyl sulphoxide

(Wako, Osaka, Japan). Aliquots of 10^6 cells in 1-ml volumes were kept at -70°C for 24 h and then transferred to a liquid nitrogen freezer.

NO-releasing agents

NO-releasing agents, S-nitroso-N-acetyl-DL-penicillamine (SNAP), 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC18), 3-{(±)-(E)-ethyl-2′-[(E)-hydroxyimino]-5-nitro-3-hexenecarbamoyl}-pyridine (NOR4) were purchased from Wako. SNAP and NOR4 were dissolved in DMSO and NOC18 was dissolved in 1 N NaOH at a concentration of 100 mM. These reagents were stored at -80°C and freshly dissolved and diluted with RPMI 1640 immediately before addition to culture. Nitrite concentrations in culture media were measured using Greiss reagent (nitrite assay kit; Cayman Chemical Co., Ann Arbor, MI). Culture medium (100 μl) containing added NO-releasing agents was mixed with an equal volume of Greiss reagent and incubated at room temperature for 10 min. Absorbance at 570 nm was measured. Nitrite concentration was calculated from nitrite standard curve.

NK and ADCC assay against VZV-infected cells

NK and ADCC assays against VZV-infected cells were performed as described previously [6]. Briefly, frozen VZV (Kawaguchi strain)-infected FS-4 cells were thawed rapidly, washed twice in RPMI 1640 with 10% FBS, and suspended in 0·1 ml RPMI 1640 with 10% FBS, then labelled with 3·7 MBq of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) per 1×10^6 cells. After 60 min incubation at 37°C in 5% CO_2 with intermittent agitation, the cells were washed and then resuspended in RPMI 1640 with 10% FBS at a concentration of 5×10^4/ml. For preincubation of VZV-infected cells with NO-releasing agents, 1×10^6 VZV-infected cells were added to confluent FS-4 cells cultured in a T-25 flask and incubated for 48 h, then NO-releasing agents were added and cultured for 24 h. VZV-infected cells were removed with 0·25% trypsin–1 mM EDTA 4 Na and resuspended in RPMI 1640 with 10% FBS and labelled with 3·7 MBq of $Na_2{}^{51}CrO_4$ (New England Nuclear) per 1×10^6 cells. Aliquots of 0·1 ml of labelled cells were added to round-bottomed microtitre plate wells. For ADCC assays, 10 μl of the aliquots of VZV^+ and VZV^- human pooled serum were added to 5×10^3 VZV-infected cells in 0·1 ml for 30 min at 4°C. The final serum dilution was 1 : 100. Aliquots of 0·1 ml of NPBMC containing 1×10^5 cells were then added. All determinations were done in triplicate. Microtitre plates were centrifuged at 500 rev/min for 3 min and incubated for 16 h at 37°C in 5% CO_2. After incubation, 0·1 ml of supernatant was harvested from each and counted in a gamma scintillation counter. Percent ^{51}Cr-release was calculated from the formula: (ct/min (experimental) − ct/min (spontaneous))/(ct/min (total) − ct/min (spontaneous)) ×100, where ct/min (total) is the release from target cells after the addition of 2% Triton X-100 to the target cells, ct/min (spontaneous) is the release from target cells incubated with medium alone, and ct/min (experimental) is the release from target cells cultured in the presence of effector cells. The percent ADCC was defined as the percent ^{51}Cr-release occurring in the presence of seropositive sera minus the percent ^{51}Cr-release occurring in the presence of seronegative sera.

A titre of IFN-α in the supernatant cultured from NPBMC with VZV-infected cells

NPBMC were cultured in plastic tubes with VZV-infected cells for 16 h at 37°C at an effector cell to target cell (E : T) ratio of 20 : 1 (1×10^6 effector cells and 5×10^4 target cells). Tubes were centrifuged at 500 ***g*** for 5 min, and the supernatant was collected and stored at -80°C. The titre of IFN-α in the supernatant was assayed by using an IFN-α RIA kit (Human IFN-α kit; Medgenics, Tokyo, Japan).

Binding assay

Effector cells (1×10^5) in 100 μl of RPMI 1640 with 10% FBS were mixed with an equal number of VZV-infected cells or VZV-infected cells preincubated with VZV^+ human pooled serum (final dilution 1 : 100) in 15-ml conical tubes. After 5 min of incubation at 37°C, the tubes were centrifuged at 500 rev/min for 5 min at room temperature. The supernatant was removed and 50 μl of RPMI 1640 were added. Cell pellets were resuspended by gentle pipetting. Cell suspension (10 μl) was removed and examined in a haemocytometer. The percentage of effector cells binding to target cells was calculated as follows: percent effector cell binding to target cells = number of effector cells binding to target cells/500 effector cells ×100.

Lymphocyte proliferation in response to VZV antigen

VZV and control antigen were prepared as described previously [9]. Aliquots of 0·1 ml of PBMC suspended in RPMI 1640 with 20% autologous serum at a concentration of 1×10^6/ml were added to each of 96 wells in flat-bottomed microtitre plates and cultured with VZV or control antigen (final dilution 1 : 100) for 6 days. Cells were labelled with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for the last 5 h of incubation. Incorporation of BrdU was measured with the cell proliferation ELISA, BrdU kit (Boehringer, Cat. no. 1 647 229; Mannheim, Germany).

Expression of CD25 on T cells cultured with VZV or control antigen

Aliquots of 1·0 ml of PBMC containing 1×10^6 cells suspended in RPMI 1640 with 20% autologous serum were cultured with VZV or control antigen (final dilution 1 : 100) in plastic tubes at 37°C in 5% CO_2 for 6 days. Surface markers of cultured cells were analysed by flow cytometry. Cells ($2–5 \times 10^5$) were incubated with 10 μl of FITC-conjugated anti-Leu-4 (CD3) and PE-conjugated anti-CD25 antibody for 30 min at 4°C. Cells were washed with Cell Wash (Becton Dickinson, Mountain View, CA) and immunofluorescein-stained cells were acquired and analysed on a FACScan (Becton Dickinson) by using the Concort 30 program (Becton Dickinson). Lymphocytes were identified by gating forward and side scatter, and verified by the use of Leukogate Simultest (Becton Dickinson).

Statistical analysis

Wilcoxon signed rank test was used for analysing samples.

RESULTS

Effects of NO-releasing agents on NK and ADCC activity against VZV-infected cells

The addition of 100 μM SNAP, NOC18 or NOR4 reduced ^{51}Cr-release from VZV-infected cells by 72·5%, 60·3%, and 99·5%, respectively. The inhibitory effects of NO-releasing agents on NK activity were dose-dependent (Table 1). The concentration of nitrite in culture medium positively correlated with inhibition of NK activity by NO-releasing agents ($r^2 = 0{\cdot}966$, $P < 0{\cdot}05$, Peason's correlation coefficient). The same concentration of DMSO and NAOH had no effect on NK activity (data not shown). ^{51}Cr-release from VZV-infected cells in the presence of

Table 1. Effects of nitric oxide (NO)-releasing agents on NK activity against VZV-infected cells

Addition of NO-releasing agents (μM)	Mean percent ^{51}Cr-release ± s.e.m. from VZV-infected cells	Concentration of nitrite in culture media (μM)
None	20·4 ± 2·9	0
SNAP (100)	5·6 ± 2·2* (72·5)†	98
SNAP (50)	13·2 ± 1·6* (35·3)	49
SNAP (20)	16·8 ± 2·0 (17·6)	25
NOC18 (100)	8·1 ± 1·2* (60·3)	83
NOC18 (50)	14·0 ± 1·6* (31·5)	41
NOC18 (20)	17·8 ± 2·3 (12·7)	16
NOR4 (100)	0·1 ± 0·6* (99·5)	136
NOR4 (50)	10·6 ± 2·5* (47·9)	64
NOR4 (20)	15·3 ± 2·2 (24·8)	33

NK assay was performed at an effector to target ratio of 20. NO-releasing agents were added to NK assay. Data are mean ± s.e.m. from five different experiments.

† Percent inhibition = (% ^{51}Cr-release without NO-releasing agents − % ^{52}Cr-release with NO-releasing agents)/(% ^{51}Cr-release without NO-releasing agents) ×100. The concentration of nitrite in the assay at the start of culture was measured by Griess reagent.

* $P < 0{\cdot}05$ compared with lack of NO-releasing agents (Wilcoxon signed rank test).

VZV$^+$ and VZV$^-$ serum was significantly inhibited by the addition of NO-releasing agents. Thus % ADCC, defined as the amount of cytotoxicity attributed to the presence of antibody, was not changed (Table 2). Pretreatment of NPBMC with 100 μM SNAP, NOC18, or NOR4 for 18 h also inhibited NK activity against VZV-infected cells. However, VZV-infected cells precultured with 100 μM SNAP, NOC18, or NOR4 were lysed, as were untreated VZV-infected cells (Table 3). Spontaneous release from VZV-infected cells was $< 25\%$ in 16 h. Spontaneous release from VZV-infected cells in the presence of SNAP, NOC18, or NOR4 was not changed.

Titre of IFN-α in the supernatant of NPBMC cultured with VZV-infected cells in the presence or absence of NO-releasing agents

Mean IFN-α titre in the supernatant of NPBMC from four different donors cultured with VZV-infected cells was 88·5 U/ml. By contrast, the mean IFN-α titre in the supernatant of cultured NPBMC in the presence of 100 μM SNAP, NOC18 or NOR4 was 12·0, 13·9, and 1·0 U/ml, respectively. Production of IFN-α was significantly inhibited by the addition of NO-releasing agents ($P < 0{\cdot}05$, Wilcoxon signed rank test). The same concentration of DMSO and NAOH had no effect on the production of IFN-α.

Effects of NO-releasing agents on effector-to-target cell binding

The percentages of effector cells binding to VZV-infected cells and VZV-infected cells preincubated with VZV$^+$ human pooled serum were 4·5% and 8·7%, respectively. The effector-to-target cell binding was unchanged by pretreatment of NPBMC with NO-releasing agents for 18 h (data not shown).

Effects of NO-releasing agents on lymphocyte proliferation and expression CD25 in response to VZV antigen

As previously reported, PBMC from VZV$^+$ donors proliferated, and the percentage of CD25 on T cells increased in response to VZV antigen. When PBMC were cultured with VZV antigen in the presence of 100 μM SNAP, NOC18 or NOR4 for 6 days, cell proliferation and the percentage of CD25 in T cells significantly decreased compared with culture with VZV antigen in the absence NO-releasing agents (Table 4).

DISCUSSION

In our study, NK activity against VZV-infected cells and the production of IFN-α by NPBMC cultured with VZV-infected

Table 2. Effects of nitric oxide (NO)-releasing agents on antibody-dependent cell-mediated cytotoxicity (ADCC) activity against VZV-infected cells

Addition of NO-releasing agents (μM)	Mean percent ^{51}Cr-release ± s.e.m. from VZV- infected cells in the presence of serum		
	Seropositive	Seronegative	% ADCC
None	47·7 ± 6·9	21·3 ± 2·5	26·4 ± 3·8
SNAP (100)	34·8 ± 6·2*	6·1 ± 2·4*	28·7 ± 4·2
SNAP (50)	37·3 ± 6·7*	12·6 ± 1·8*	24·7 ± 3·8
SNAP (20)	42·9 ± 5·9	18·2 ± 1·6	24·7 ± 3·6
NOC18 (100)	36·2 ± 5·8*	9·1 ± 0·6*	27·1 ± 4·2
NOC18 (50)	38·1 ± 6·3*	15·2 ± 2·5*	22·9 ± 3·7
NOC18 (20)	40·8 ± 6·6	18·2 ± 2·8	22·6 ± 3·2
NOR4 (100)	26·5 ± 6·0*	0·5 ± 0·8*	26·0 ± 4·1
NOR4 (50)	30·3 ± 4·9*	9·2 ± 1·7*	21·1 ± 3·3
NOR4 (20)	34·9 ± 5·4	12·3 ± 2·4	22·6 ± 2·9

ADCC assay was performed at an effector to target ratio of 20. NO-releasing agents were added to ADCC assay. Data are mean ± s.e.m. from five different experiments.

* $P < 0{\cdot}05$ compared with lack of NO-releasing agents (Wilcoxon signed rank test).

Table 3. Effects of pretreatment of effector cells and VZV-infected cells with nitric oxide (NO)-releasing agents on NK activity against VZV-infected cells

Treatment of effector cells	Treatment of VZV-infected cells	Mean percent ^{51}Cr-release ± s.e.m. from VZV-infected cells
None	None	25·8 ± 3·5
SNAP	None	15·2 ± 2·7*
NOC18	None	14·8 ± 2·6*
NOR4	None	9·5 ± 3·1*
None	SNAP	24·6 ± 2·1
None	NOC18	25·3 ± 1·9
None	NOR4	27·4 ± 2·2

Non-adherent peripheral blood mononuclear cells (NPBMC; 1×10^6) and VZV-infected cells were cultured with 100 μM SNAP, NOC18, NOR4 for 18 h. After culture, the cells were washed three times and NK assay against VZV-infected cells was performed at an effector cell to target cell ratio of 20. Data are mean ± s.e.m. from four different experiments.

* $P < 0{\cdot}05$ compared with lack of NO-releasing agents (Wilcoxon signed rank test).

cells were significantly inhibited by the addition of NO-releasing agents to assay media. The mechanisms by which NO inhibits NK activity against VZV-infected cells are unclear. NO had no effect on the susceptibility of VZV-infected cells to NK cells because preincubation of VZV-infected cells by NO-releasing agents had no effect on the killing of VZV-infected cells. It is unlikely that NO-releasing agents had toxic effects on effector cells, because the viability of NPBMC treated with NO-releasing agents was > 97%, as determined by trypan blue exclusion. Binding of effector cells to target cells was also unaffected by treatment of NPBMC with NO-releasing agents. These data suggest that NO affects the killing pathway after effector-to-target cell binding. It was reported that NK cell-mediated lysis of virus-infected cells required the production of IFN-α by non-adherent accessory cells [10]. Inhibition of the production of IFN-α by NO-releasing agents may affect NK activity against VZV-infected cells.

T cell activation by VZV antigen was also inhibited by the addition of NO-releasing agents in culture media. It was reported that NO inhibits mitogen-stimulated proliferation of lymphocytes. Fu & Blankenhorn [11] reported that NO produced by macrophages inhibited the response of rat spleen cells to mitogens, but did not inhibit IL-2 production. Lepoivre *et al.* [12] demonstrated that NO inhibits the activity of ribonucleotide reductase, an enzyme necessary for DNA synthesis. However, the mechanism by which NO inhibits T lymphocyte proliferation has not been determined. Some intracellular signal pathway required for proliferation and cytotoxicity may be affected by NO. Further studies on changes of intracellular pathway in NK cells and activated T cells by NO are needed.

NO is released from endothelial cells, vascular smooth muscle, and neutrophils in response to IFN-γ, TNF-α, IL-1 and endotoxin [4,5]. It has been proven that NO is induced in humans. Devlin *et al.* [13] reported that the plasma concentration of NO increased during acute allograft rejection and was correlated with liver allograft rejection, and Ochoa *et al.* [14] described NO plasma levels of 32·3 μM in patients with cancer and a nine-fold increase of NO after human tumour immunotherapy. NO can be produced *in vivo* at a concentration used in this experiment. Recently, it was reported that CD69 cross-linking induced NO production from human monocytes [15]. These results suggest that excessive production of NO at inflammatory sites may affect the host defence immune reaction. Our findings suggest that the production of NO by an inflammatory process may lead to inhibition of NK cell- and T cell-mediated immunity to VZV infection.

Table 4. The effects of nitric oxide (NO)-releasing agents on lymphocyte proliferation and expression of CD25 in response to VZV antigen

Antigen	Addition of NO-releasing agents (μM)	Cell proliferation of PBMC (mean) absorbance ± s.e.m)	Mean percentage of CD25$^+$ cells in T cells (± s.e.m.)
VZV	None	0·703 ± 0·04	41·0 ± 4·2
VZV	SNAP (100)	0·465 ± 0·03*	29·4 ± 2·6*
VZV	NOC18 (100)	0·324 ± 0·07*	28·3 ± 3·7*
VZV	NOR4 (100)	0·337 ± 0·02*	28·6 ± 1·5*
Control	None	0·008 ± 0·01	14·3 ± 3·0

Peripheral blood mononuclear cells (PBMC) from five VZV$^+$ adults were cultured with VZV or control antigen for 6 days. NO-releasing agents were added to culture. Data are mean ± s.e.m. from five different experiments.

* $P < 0{\cdot}05$ compared with lack of NO-releasing agents (Wilcoxon signed rank test).

REFERENCES

1 Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 1988; **336:**385–8.

2 Stuehr DJ, Nathan CF. Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 1989; **169:**1543–55.

3 Granger DL, Hibbs J Jr, Perfect JR *et al.* Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. J Clin Invest 1988; **81:**1129–36.

4 Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. J Natl Cancer Inst 1990; **82:**772–6.

5 Wright CD, Mulsch A, Busse R *et al.* Generation of nitric oxide by human neutrophils. Biochem Biophys Res Commun 1989; **160:**813–9.

6 Ito M, Bandyopadhyay S, Matsumoto-Kobayashi M *et al.* Interleukin 2 enhances natural killing of varicella-zoster virus-infected targets. Cli Exp Immunol 1986; **65:**182–9.
7 Ito M, Nakano T, Kamiya T *et al.* Activation of lymphocytes by varicella-zoster virus (VZV): expression of interleukin-2 receptors on lymphocytes cultured with VZV antigen. J Infect Dis 1992; **165:**158–61
8 Kumagai K, Itoh K, Hinuma S *et al.* Pretreatment of plastic Petri dishes with fetal calf serum. A simple method for macrophage isolation. J Immunol Methods 1979; **29:** 17–25.
9 Zaia JA, Leary PL, Levin ML. Specificity of the blastogenic response of human mononuclear cells to herpesvirus antigen. Infect Immun 1978; **20:**646–51.
10 Bandyopadhyay S, Perussia B, Trinchieri G *et al.* Requirement for HLA-DR$^+$ accessory cells in natural killing of cytomegalovirus-infected fibroblasts. J Exp Med 1986; **164:**180–95.
11 Fu Y, Blankenhorn EP. Nitric oxide-induced anti-mitogenic effects in high and low responder rat strains. J Immunol 1992; **148:**2217–22.
12 Lepoivre M, Chenais B,Yapo A *et al.* Alterations of ribonucleotide reductase activity following induction of the nitrite-generating pathway in adenocarcinoma cells. J Biol Chem 1990; **265:** 14143–9.
13 Devlin J, Palmer RM, Gonde CE *et al.* Nitric oxide generation. A predictive parameter of acute allograft rejection. Transplantation 1994; **58:**592–5.
14 Ochoa JB, Curti B, Peitzman AB *et al.* Increased circulating nitrogen oxides after human tumor immunotherapy: correlation with toxic hemodynamic changes. J Natl Cancer Inst 1992; **84:** 864–7.
15 De Maria R, Cifone MG, Trotta R *et al.* Triggering of human monocyte activation through CD69, a member of the natural killer cell gene complex family of signal transducing receptors. J Exp Med 1994; **180:**1999–2004.