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Inhibition of human lymphocyte proliferation by nitric oxide-releasing oxatriazole derivatives

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Abstract

The effects of novel nitric oxide (NO)-releasing oxatriazole derivatives GEA 3162 and GEA 3175 were studied on cell proliferation and cGMP synthesis in human peripheral blood mononuclear cells stimulated with a lectin mitogen concanavalin A. GEA 3162 (1–30 μM) and GEA 3175 (3–30 μM) inhibited mononuclear cell proliferation in a dose-dependent manner being more potent than the earlier known NO-donor *S*-nitroso-*N*-acetylpenicillamine. The inhibitory action was more pronounced when submaximally stimulating concentrations of concanavalin A (0.1 and 1 μg/ml) were used and no inhibition was seen when concanavalin A concentrations were increased up to 10 μg/ml. The antiproliferative concentrations of GEA 3162, GEA 3175 and *S*-nitroso-*N*-acetylpenicillamine induced a rapid and transient increase in cGMP production in mononuclear cells cultured in the presence of concanavalin A. Both the antiproliferative action and the increased cGMP production were attenuated when red blood cells were added into the cultures indicating that NO is responsible for both of these actions. An analogue of cGMP, 8-bromo-cGMP (0.1–3 mM) reduced concanavalin A-induced proliferation in a dose-dependent manner suggesting that cGMP may be involved in the antiproliferative action of NO-donors. NO-releasing compounds have immunosuppressive actions which offer therapeutic possibilities and should be kept in mind as potential adverse events when these compounds are used in other indications. © 1997 Elsevier Science B.V.

Keywords: Nitric oxide (NO)-releasing compound; Nitric oxide (NO); cGMP; Lymphocyte

1. Introduction

Nitric oxide (NO) is a labile substance which equals the activity of endothelium-derived relaxing factor and causes vasodilatation by relaxing vascular smooth muscle. In addition, NO acts as a signalling molecule in functions as diverse as neural communication, immunomodulation and regulation of platelet activation (Moncada et al., 1991; Moncada, 1992; Moilanen and Vapaatalo, 1995). Production of large amounts of NO by activated macrophages accounts for their antimicrobial activity. There is also data supporting the regulatory role of NO produced by activated macrophages or tumor cells on lymphocyte function (Mills, 1991; Albina et al., 1991; Lejeune et al., 1994). Using murine T-cell lines Taylor-Robinson et al. (1994) found that T-helper type 1 (T_h1) but not T-helper type 2 (T_h2) cells produced large amounts of NO which serves as a regulatory mechanism leading to suppressed function of T_h1 lymphocytes. Based on these findings, a hypothesis on a profound NO-induced regulation of immune response in inflammatory diseases like asthma has been suggested (Barnes and Liew, 1995).

NO-donors have therapeutic potential in a range of pathologic conditions (Moncada and Higgs, 1995). Organic nitrates exert their pharmacological actions by releasing NO in enzymatic and nonenzymatic processes taking place mainly in vascular endothelium (Feelisch, 1993). Recently, groups of structurally different molecules able to release NO spontaneously or after enzymatic conversion have been developed. GEA 3162 and GEA 3175 are mesoionic 3-aryl substituted oxatriazole-5-imine derivatives known to release NO in aqueous solutions (Karup et al., 1994; Kankaanranta et al., 1996). We have earlier found that these compounds inhibit neutrophil functions (Moilanen et al., 1993), leukocyte adhesion to endothelial cells (Moilanen et al., 1994) and suppress tumour cell growth (Vilpo et al., 1994). These NO-donors have also vasodilator, antiplatelet, fibrinolytic (Corell et al., 1994) and antibacterial (Virta et al., 1994) activities.

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NO seems to have immunosuppressive effects which offer therapeutic possibilities and should be kept in mind as potential adverse effects of NO-releasing compounds. The present study was designed to evaluate the effects of NO-releasing oxatriazole-5-imine derivatives on human lymphocyte proliferation.

2. Materials and methods

2.1. Cell isolation and proliferation assay

Human peripheral blood mononuclear cells were isolated by Ficoll-Paque gradient centrifugation from venous blood obtained from healthy volunteers who had abstained from any drugs for at least one week before sampling. The cell suspension consisted of lymphocytes ($90.0 \pm 1.7\%$), monocytes ($7.4 \pm 1.5\%$) and polymorphonuclear leukocytes ($2.6 \pm 0.5\%$) (mean $\pm$ S.E.M., $n = 7$). The cells were suspended in RPMI 1640 Glutamax-1 supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml). Lymphocyte proliferation was induced by concanavalin A. The cells were cultured in 96-well plates (2×10^5/200 μl). NO-donors, red blood cells and other compounds tested were added into the culture just before concanavalin A. The cells were incubated for 2 days at 37°C (in 5% CO_2) and then pulsed for 20 h with 0.1 μCi [^{3}H]thymidine. The cells were harvested and the incorporated radioactivity was measured by β-counter. To evaluate a direct cytotoxicity of the NO-donors, trypan blue staining and measurement of released lactate dehydrogenase were included in the protocol. None of the NO-donors tested decreased cell viability as measured by these tests in the incubation conditions described above.

2.2. Determination of cyclic guanosine 3′:5′-monophosphate (cGMP) production

Peripheral blood mononuclear cells (5×10^6 cells in 500 μl of RPMI) were incubated with concanavalin A 1 μg/ml and the NO-donor for the time indicated at 37°C. The incubations were terminated by addition of ice cold trichloroacetic acid (final concentration 6%) and the samples were centrifuged ($10\,000 \times g$ for 5 min). The supernatants were washed four times with water-saturated ether, diluted with an equal volume of 100 mM sodium acetate buffer (pH 6.2) and stored at −20°C until assayed for cGMP. The cGMP samples were acetylated and measured by radioimmunoassay as described earlier (Axelsson et al., 1988; Moilanen et al., 1993).

2.3. Drugs and chemicals

The two mesoionic 3-aryl-substituted oxatriazole-5-imine derivatives GEA 3162 and GEA 3175 as well as *S*-nitroso-*N*-acetylpenicillamine were kindly provided by GEA (Copenhagen, Denmark). Culture media and media supplements (Gibco, Paisley, UK), concanavalin A and Ficoll-Paque (Pharmacia, Uppsala, Sweden), ^{125}I-labelled cyclic GMP (DuPont, Boston, MA, USA) and [methyl-^{3}H]thymidine (Amersham International, Amersham, UK) were obtained as indicated.

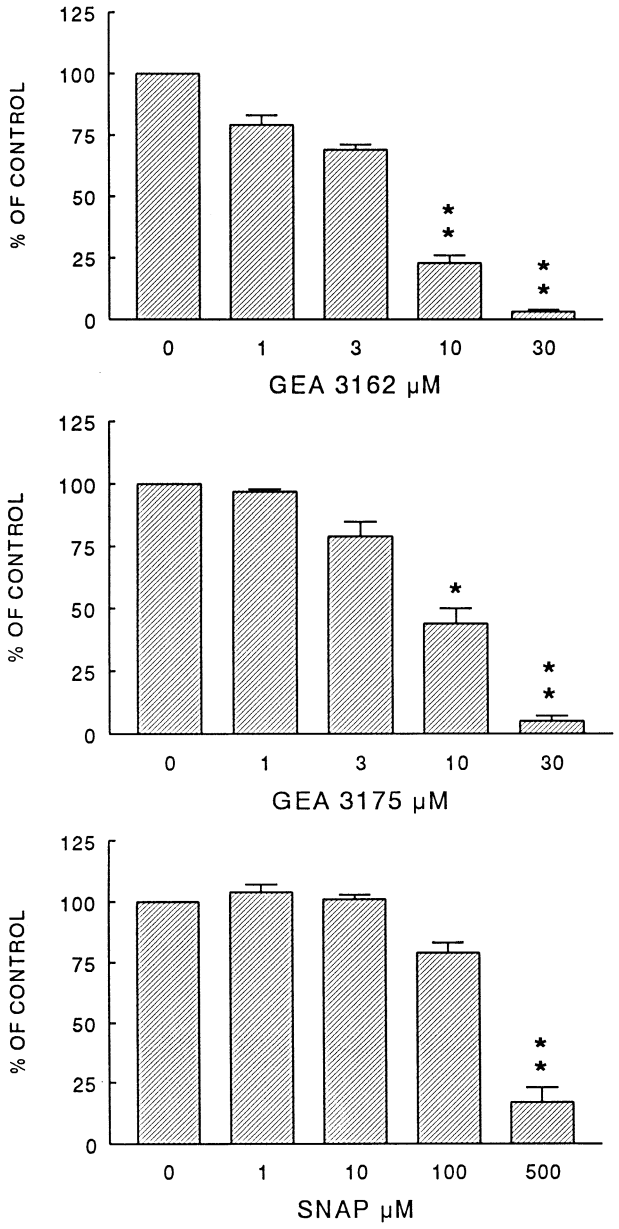

Fig. 1. The inhibitory action of GEA 3162, GEA 3175 and *S*-nitroso-*N*-acetylpenicillamine (SNAP) on concanavalin A-induced proliferation of human mononuclear cells. The cells were cultured for 68 h in the presence of concanavalin A 1 μg/ml and NO-donor. The cell proliferation was determined by the uptake of [^{3}H]thymidine (0.1 μCi/well), which was added 20 h before the end of culture. Results are expressed as percent of control (i.e., cells stimulated by concanavalin A in the absence of NO-donors). Values are the mean ± S.E.M. of six triplicate experiments. * $P < 0.05$ and ** $P < 0.01$ versus control without NO-donor.

2.4. Statistics

Results are expressed as mean ± S.E.M. Statistical significance was calculated by analysis of variance for repeated measures supported by Dunnett's multiple comparisons test and by Friedman nonparametric repeated measures test followed by Dunn's multiple comparisons test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Effects of NO-donors on human mononuclear cell proliferation

The three NO-donors inhibited in a dose-dependent manner the proliferation of human mononuclear cells stimulated with submaximal concentrations (1 μg/ml) of concanavalin A. On a molar basis the two new oxatriazole derivatives (GEA 3162 and GEA 3175) were more potent than the earlier known NO-releasing compound *S*-nitroso-*N*-acetylpenicillamine (Fig. 1). Lower concentrations of NO-donors (GEA 3162 and GEA 3175 1–100 nM and *S*-nitroso-*N*-acetylpenicillamine 10–100 nM) had no effect on mononuclear cell proliferation. The inhibitory action was most pronounced when submaximally stimulating concentrations of concanavalin A (0.1 and 1 μg/ml) were used and was reversed when concanavalin A concentrations were increased (Fig. 2).

3.2. Effects of NO-donors on cGMP synthesis in human peripheral blood mononuclear cells

One of the cellular effects of NO is to activate the enzyme guanylate cyclase which converts GTP to an active intracellular second messenger cGMP (Ignarro, 1991; Moncada et al., 1991). The NO-donors used induced an increase in cGMP levels in human mononuclear cells after 30 min and 2 h exposure to the NO-donor (Table 1). The time course of the NO-donor-induced cGMP production was somewhat different between the three compounds tested. Cyclic GMP levels induced by GEA 3162 were higher after 30 min than after 2 h incubation, whereas cGMP levels after GEA 3175 and *S*-nitroso-*N*-acetylpenicillamine continued to increase when the incubation time was prolonged from 30 min to 2 h. When the NO-donors were incubated with concanavalin A-activated mononuclear cells for 24 h, cGMP levels comparable to the pre-treatment values were found.

Table 1
NO-donor induced production of cGMP (fmol/10^6 cells) in concanavalin A-stimulated human peripheral blood mononuclear cells

	30 min	2 h
Without NO-donor	81 ± 22	95 ± 18
GEA 3162 3 μM	859 ± 232	507 ± 96
GEA 3162 30 μM	2351 ± 735 *	1195 ± 270 *
GEA 3175 3 μM	251 ± 48	431 ± 119
GEA 3175 30 μM	521 ± 181 *	1344 ± 671 *
SNAP 100 μM	2457 ± 882 **	3301 ± 1266 **
SNAP 500 μM	2981 ± 1169 ***	4330 ± 1117 ***

The cells were incubated at 37°C with concanavalin A 1 μg/ml and the NO-donor. The incubations were stopped by addition of cold trichloroacetic acid. The results are expressed as mean ± S.E.M. of six duplicate experiments.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ as compared with control without NO-donor.

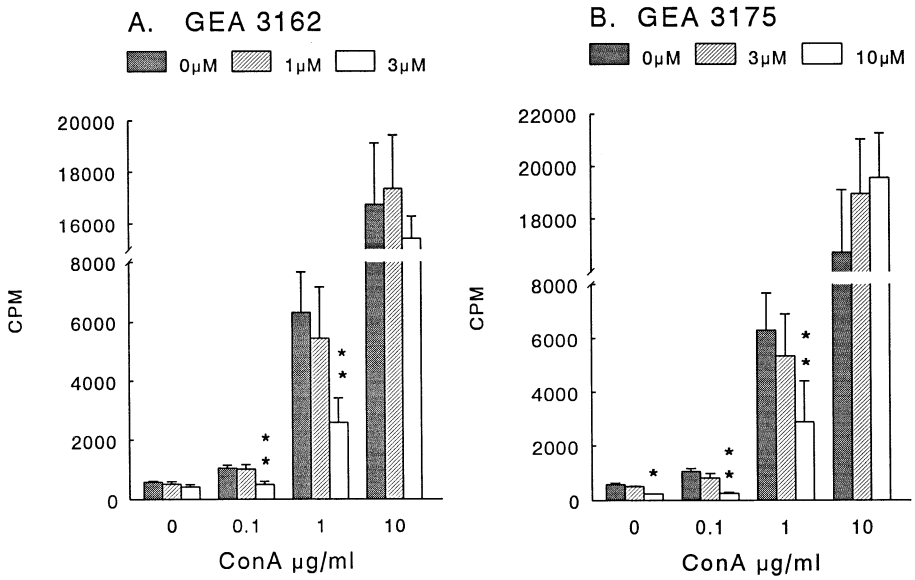

Fig. 2. The effects of GEA 3162 and GEA 3175 on concanavalin A-induced proliferation of human mononuclear cells. The dose–response curve of concanavalin A is shown. The results are expressed as mean [^{3}H]thymidine incorporation ± S.E.M. of four triplicate experiments. * $P < 0.05$ and ** $P < 0.01$ versus respective control without NO-donor.

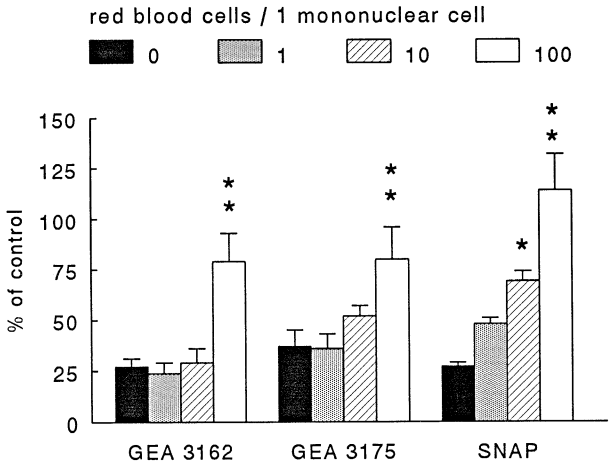

Fig. 3. The effects of red blood cells on the action of GEA 3162 (10 μM), GEA 3175 (10 μM) and *S*-nitroso-*N*-acetylpenicillamine (SNAP; 500 μM). Human mononuclear cell proliferation was determined by [^{3}H]thymidine incorporation. The cells were cultured with concanavalin A (1 μg/ml) and NO-donor for 68 h either in the absence or presence of red blood cells. The results are expressed as percent of control (i.e., the cells cultured without NO-donor). The values are the mean ± S.E.M. of four triplicate experiments. * $P < 0.05$ and ** $P < 0.01$ as compared with the respective control without red blood cells.

3.3. Effect of red blood cells on the actions of NO-donors

The addition of red blood cells into the culture reversed the inhibitory effect of NO-donors on lymphocyte proliferation (Fig. 3). The action of *S*-nitroso-*N*-acetylpenicillamine seemed to be the most sensitive to the action of red blood cells. Increased cGMP production in mononuclear cells incubated for 30 min in the presence of NO-donors was attenuated by 42–47% when red cells (10 red cells/1 mononuclear cell) were added into the cultures.

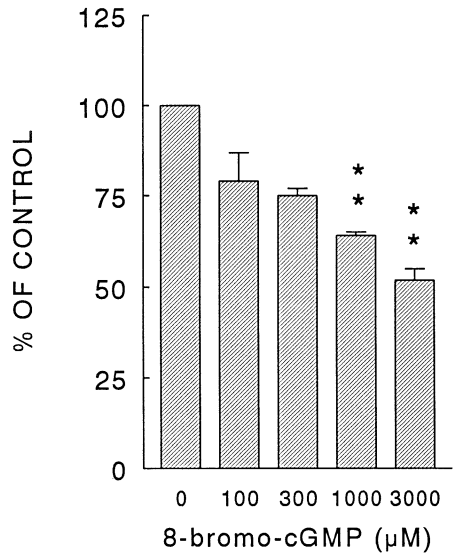

Fig. 4. The effect of an analogue of cGMP, 8-bromo-cGMP on concanavalin A-induced proliferation of human mononuclear cell. The cell proliferation was determined by uptake of [^{3}H]thymidine. Results are expressed as percent of control (i.e., cells stimulated by concanavalin A in the absence of 8-bromo-cGMP). Values are the mean ± S.E.M. of 3–6 triplicate experiments. ** $P < 0.01$ versus control without 8-bromo-cGMP.

3.4. Effect of 8-bromo-cGMP on human peripheral blood mononuclear cell proliferation

The effects of an analogue of cGMP, 8-bromo-cGMP were tested on lymphocyte proliferation. 8-Bromo-cGMP (0.1–3 mM) inhibited concanavalin A (1 μg/ml)-induced proliferation in a dose-dependent manner resulting in a 36% and 48% reduction at 1 and 3 mM concentration, respectively (Fig. 4).

4. Discussion

In the present work, we demonstrate that NO-releasing compounds inhibit proliferative responses in human peripheral blood mononuclear cells stimulated with a lectin mitogen concanavalin A. Two novel oxatriazole-5-imine derivatives GEA 3162 and GEA 3175 were more potent than the earlier known compound *S*-nitroso-*N*-acetylpenicillamine.

NO-releasing properties of GEA 3162 and GEA 3175 have been recently characterized by documenting their ability to inhibit platelet aggregation, induce cGMP synthesis in platelets, convert oxyhemoglobin to methemoglobin, generate nitrite and nitrate in aqueous solutions and to form nitrosyl–hemoglobin complex (Karup et al., 1994; Kankaanranta et al., 1996). GEA 3162 and GEA 3175 have been shown to have vasodilator, antiplatelet, fibrinolytic (Corell et al., 1994) and antibacterial (Virta et al., 1994) activities as well as to inhibit neutrophil functions (Moilanen et al., 1993, 1994), suppress tumour cell growth (Vilpo et al., 1994), regulate glycosaminoglycan synthesis in articular cartilage (Järvinen et al., 1995) and to inhibit oxidation of low density lipoprotein (Malo-Ranta et al., 1994).

The kinetics of NO-release from GEA compounds has been studied by following the formation of nitrite and nitrate in buffer solutions and by measuring E.P.R. signals of nitrosylhemoglobin of venous blood treated with these NO-donors (Kankaanranta et al., 1996). In venous blood, GEA 3162 and GEA 3175 gave rise to a nitrosylhemoglobin-like E.P.R. signal in minutes. During a 2 h follow-up time the amplitude of this signal was not markedly increased suggesting a rapid release of NO. When GEA 3162 was incubated in buffer solution, the concentration of nitrite + nitrate detected after 60 min incubation was 50% of the original concentration of the NO-donor (Kankaanranta et al., 1996). The kinetics of the increased production of cGMP was measured in human neutrophils treated with GEA compounds (Moilanen et al., 1993). When the cells were incubated with GEA 3162, cGMP levels peaked during 10 min incubations and reduced thereafter whereas cGMP levels in cells treated with *S*-nitroso-*N*-acetylpenicillamine continued to increase dur-

ing the 90 min follow-up. The present data on the kinetics of cGMP production in mononuclear cells treated with GEA NO-donors support these earlier findings that the release of NO from these two GEA compounds is rapid in onset and peaks in the early phases of the incubations, GEA 3162 being more rapid NO-releaser than GEA 3175 and *S*-nitroso-*N*-acetylpenicillamine.

In the present study, both the antiproliferative action and the increased cGMP synthesis by NO-donors was attenuated when red blood cells were added into the culture. Hemoglobin inhibits the action of NO by binding it to form nitrosylhemoglobin or by metabolising it to inactive nitrate (Murphy and Noack, 1994). Therefore the reduced action of NO-donors in the presence of red cells suggests that NO is involved in the anti-proliferative action of these compounds. These data are further supported by the earlier findings that NO produced by activated macrophages or released from *S*-nitrosoglutathione inhibits T-lymphocyte proliferation in vitro (Hoffman et al., 1990; Albina et al., 1991; Mills, 1991; Merryman et al., 1993) and in some experimentally induced infections in vivo (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993). In addition, Lander et al. (1993a,b, 1995) reported that NO at low concentrations stimulates lymphocytes through activation of $p21^{ras}$ and nuclear factor-κB. Therefore, we tested the effects of a wide concentration range of the NO-releasing compounds and only antiproliferative action was found.

The antiproliferative action of the NO-donors found in the present study was most pronounced when the cells were stimulated with submaximal concentrations of concanavalin A (0.1 and 1 μg/ml). When concanavalin A concentrations were increased up to 10 μg/ml, the antiproliferative action of NO-donors was reversed. These data together with the finding that trypan blue staining and lactate dehydrogenase release was not affected by the treatment with NO-donors suggest that the NO-donors in these concentrations have reversible and regulatory rather than cytotoxic effect on concanavalin A-activated mononuclear cells.

At physiological concentrations of NO, the enzyme guanylate cyclase is the principal target of NO (Ignarro, 1991). Increased synthesis of cGMP mediates the vasodilatory and antiaggregatory actions of NO as well as its effects on neurotransmission. In this study NO-donors caused a rapid and transient increase in cGMP production in mononuclear cells. During 30 min to 2 h incubation with the NO-donors, cGMP-levels in mononuclear cells were increased substantially. Since the early events are critical in the regulation of mitogen-induced lymphocyte proliferation, the time course of the increase in cGMP concentration is compatible with the hypothesis that antiproliferative actions of NO-donors are mediated by cGMP. However, there was no clear correlation between the degree of the antiproliferative action of NO-donors and their ability to raise cGMP levels measured at these time points. A cell-permeable analogue of cGMP, 8-bromo-cGMP, inhibited lymphocyte proliferation in a dose-dependent manner, but even in 3 mM concentrations 8-bromo-cGMP reached ca. 50% inhibition whereas NO-donors inhibited lymphocyte proliferation more profoundly. The present data gives some support on the mediator role of cGMP in the antiproliferative action of NO-donors. The lack of clear correlation between cGMP levels and the antiproliferative action suggest the presence of more complex mechanisms.

In addition to guanylate cyclase NO has direct effects on various other enzymes. For instance, NO has been reported to inactivate ribonucleotide reductase and thus DNA synthesis in tumour cells (Lepoivre et al., 1990; Kwon et al., 1991). This is also a possible explanation for the antiproliferative action of NO-donors. Another possibility arises from the finding that NO inhibits Ia antigen expression on antigen presenting cells (Sicher et al., 1994) since concanavalin A-stimulation is dependent on major histocompatibility complex (MHC) II expression in antigen presenting cells (Ahmann et al., 1978). The non-cGMP actions of NO and related species may be mediated by *S*-nitrosylation of protein sulfhydryl groups (Stamler et al., 1997).

The significance of endogenously produced NO as a regulator of human lymphocyte responses is presently not known. Murine lymphocytes produce NO in a subtype-specific manner (Taylor-Robinson et al., 1994). This has been suggested to serve as an auto-regulatory mechanism leading to suppressed function of T_h1 lymphocytes (Barnes and Liew, 1995). NO synthesis in human lymphocytes has been difficult to demonstrate. Mannick et al. (1994) reported an expression of a constitutive, low level, macrophage-type NO synthase in Epstein–Barr virus-transformed human B lymphocytes and Burkitt's lymphoma cell lines. In addition, the induction of NO synthesis in human monocyte-macrophages has been described (Denis, 1991; De Maria et al., 1994; Mossalayi et al., 1994; Bukrinsky et al., 1995; Vouldoukis et al., 1995; Moilanen et al., 1997). Although the regulatory role of endogenously produced NO in human immune system remains obscure, the present data show that exogenous NO has an antiproliferative action on human peripheral blood lymphocytes. The effect was attenuated in the presence of erythrocytes suggesting that lymphocytes accumulated, for example, into an inflammatory focus may be more susceptible for the action of NO-releasing compounds than cells in the circulation or in the spleen. The immunosuppressive action of NO-releasing compounds offers therapeutic possibilities and may lead to adverse effects when NO-releasing compounds are used for other indications

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