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The role of nitric oxide as an effector of macrophage-mediated cytotoxicity against *Trichomonas vaginalis*

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Abstract: The purpose of this study is to determine whether nitric oxide is involved in the extracellular killing of *Trichomonas vaginalis* by mouse (BALB/c) peritoneal macrophages and RAW264.7 cells activated with LPS or rIFN-γ and also to observe the effects of various chemicals which affect the production of reactive nitrogen intermediates (RNI) in the cytotoxicity against *T. vaginalis*. The cytotoxicity was measured by counting the release of [^{3}H]-thymidine from labelled protozoa and NO_2^- was assayed by Griess reaction. N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (NAME) and arginase inhibited cytotoxicity to *T. vaginalis* and nitrite production by activated mouse perioneal macrophages and RAW 264.7 cells. The addition of excess L-arginine competitively restored trichomonacidal activity of macrophages. Exogenous addition of $FeSO_4$ inhibited cytotoxicity to *T. vaginalis* and nitric products of macrophages. From above results, it is assumed that nitric oxide plays an important role in the host defense mechanism of macrophages against *T. vaginalis*.

Key words: Nitric oxide, mouse peritoneal macrophage, RAW264.7 cell, *Trichomonas vaginalis*, cytotoxicity, nitrite production

INTRODUCTION

Macrophages have been implicated as an effector cell of human protective immunity as natural killer cells. The activated macrophages release various enzymes, tumor necrosis factor (TNF), reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI; NO, NO_2^-, NO_3^-), and these products demonstrate toxicity toward intracellular and extracellular targets (James, 1991).

Currently there has been much interest in the biological role of nitric oxide. Nitric oxide is derived from oxidation of the N-terminal guanidino nitrogen atoms of L-arginine and also generates citrulline (James and Hibbs, 1990). Macrophages activated with IFN-γ and LPS *in vitro* produce large amount of NO (Liew, 1991; Cunha *et al.*, 1993). Nitric oxide appears to play an important function as an effector molecule to tumor cells (Hibbs *et al.*, 1987) and various parasites such as *Cryptococcus* (Granger *et al.*, 1989), *Toxoplasma gondii* (Adams *et al.*, 1990), *Schistosoma mansoni* (James and Glaven, 1989) and *Entamoeba histolytica* (Lin and Chadee, 1992) in cytotoxicity.

Natural cell-mediated cytotoxicity against extracellular parasites *T. vaginalis*, is mediated by macrophages *in vitro*, and cytotoxicity by lymphokine-activated peritoneal macrophages is much increased than non-activated

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peritoneal macrophages (Landolfo *et al.*, 1980; Ryu *et al.*, 1990 & 1995; Yoon *et al.*, 1991). Until now no definite evidence has been demonstrated in association between cytotoxicity and nitrite production by activated macrophages against *T. vaginalis*.

Our studies were designed to determine whether activated BALB/c mouse peritoneal macrophages and RAW264.7 cells derived from mouse peritoneal tumor cells have cytotoxicity against extracellular parasites, *T. vaginalis*, and whether NO was involved in the cytotoxicity against *T. vaginalis*.

MATERIALS AND METHODS

Reagents and experimental animals

Arginase, ferrous sulfate, and lipopolysaccharide (LPS, from *Escherichia coli* K-235) were purchased from Sigma (MO, USA), rIFN-γ (specific activity 1.1×10^7 unit/mg) from Genzyme (Maine, USA); N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (NAME), (6R)-5, 6, 7, 8-tetrahydro-L-biopterin (H_4B) from RBI (Research Biochemicals International, MA, USA).

BALB/c mice, 18~20 g which have been raised in Department of Parasitology, College of Medicine, Hanyang University, were used in the present study.

Trichomonas vaginalis culture

T. vaginalis KT9 isolate was isolated from the vaginal secretion of a Korean woman with acute vaginitis. Trophozoites were axenically cultured in Diamond s TYM medium (1957).

Macrophages

Two types of macrophages were used in this experiment. The murine macrophage cell line RAW264.7 was purchased from the American Tissue Culture Collection (ATCC). This line was established from the asites of a tumor induced in a male mouse by the intraperitoneal injection of Abelson leukemia virus (A-MuLV).

Murine peritoneal macrophages (MPMs) were harvested from BALB/c mice which had been injected intraperitoneally 3 days previously with 1 ml of 10% proteose peptone and were cultured for 2 hr at 37°C in an atmosphere of 5% CO_2 in Costar plate at 5×10^6 cells. Nonadherent cells were removed by washing with pre-warmed (37°C) medium. Macrophages were activated *in vitro* by addition of IFN-γ (100 U/ml) or LPS (20 ng/ml) for 24 hr in DMEM media.

Cytotoxicity assay

For cytotoxicity assay *T. vaginalis* was seeded at a density of 5×10^5 cell/ml in 2 ml of 40:1 (vol/vol) DMEM/TYM supplemented with 30 μCi methyl-[^{3}H]-thymidine (specific activity 25 Ci/mmol, Amersham, UK). After incubating for 24 hr at 37°C in shaking water bath, trichomonads were washed and resuspended in test media.

In case of mouse peritoneal macrophages, 0.1 ml of a suspension of labeled protozoa (2×10^4) was placed in each well of U-bottom 96-well plate, followed by 0.1 ml of a suspension containing 1.4×10^5 effector macrophages.

RAW264.7 cells (10^5) and 2×10^4 labeled trichomonads were mixed in each well of 24-well plate containing 0.5 ml DMEM. Both plates were incubated at 37°C in 5% CO_2 incubator for 18 h, and plates were centrifuged at 400 g for 10 min, and 0.1 ml of the supernatants was collected from each well and counted in scintillation counter (PACKARD, USA). The cytotoxicity was calculated as below:

$$\% \text{ Cytotoxicity} = 100 \times \frac{\text{cpm of experimental release} - \text{cpm of spontaneous release}}{\text{cpm of maximum release} - \text{cpm of spontaneous release}}$$

Measurement of NO_2^- production

Since NO rapidly degrades to NO_2^-, NO_3^- under aerobic aqueous conditions, measurement of these intermediates are routinely used for determination of the reactivity. In the present study, we measured NO_2^- by Griess reaction (Yoon *et al.*, 1991).

Briefly, an equal volume of Griess reagent (1% sulfanilamide/0.1% N- (1-naphthyl) ethylenediamine dihydrochloride/2.5% H_3PO_4) was incubated with macrophage culture supernatants for 10 min at room temperature

and absorbance was measured at 570 nm in ELISA reader. Nitrite concentration was determined using $NaNO_2$ as standard.

RESULTS

Effects of rIFN-γ and/or LPS on the cytotoxicity to *T. vaginalis*

When MPMs and RAW264.7 cells were activated with rIFN-γ and/or LPS, the cytotoxicity of two different macrophges showed different patterns, but nitrite productions showed similar pattern each other. The cytotoxicity of MPMs was increased after LPS stimulation in comparison with that of MPMs without rIFN-γ and/or LPS. On the other hand, cytotoxicity of RAW264.7 cells was augmented after treatment with LPS, rIFN-γ, rIFN-γ plus LPS. Nitrite productions were significantly increased when both of macrophages were activated with rIFN-γ or rIFN-γ plus LPS as compared with macrophages without treatment (Fig. 1). In order to observe the effect of various chemicals on the cytotoxicity to *T. vaginalis* and nitrite production, MPMs and RAW264.7 cells were activated with LPS and rIFN-γ, respectively. In case of mouse peritoneal macrophges, LPS-activated MPMs showed increased cytotoxicity although LPS-activated MPMs did not produce appreciable amount of nitrite.

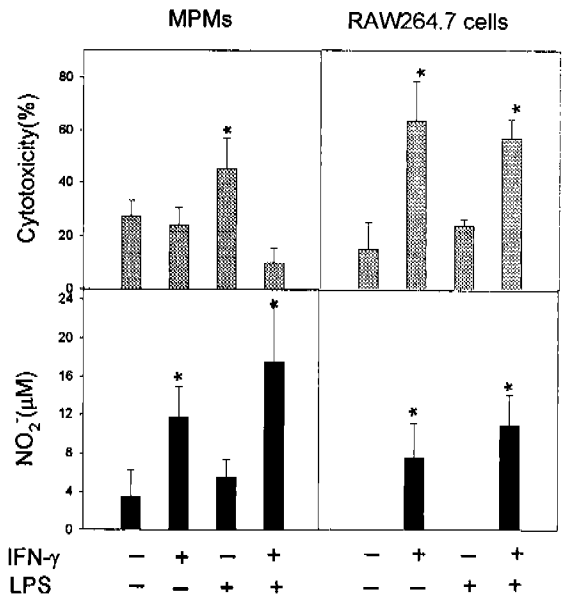

Fig. 1. Effects of IFN-γ and/or LPS on the cytotoxicity to *T. vaginalis* and nitrite production by mouse peritoneal macrophages (MPMs) or RAW264.7 cells. Control groups are MPMs and RAW264.7 cells without IFN-γ or LPS. Results by macrophages activated with IFN-γ and/or LPS, were compared with that of control macrophages. *p < 0.005, Mann-Whitney test

Effects of L-arginine analogues and arginase on the cytotoxicity to *T. vaginalis* and nitrite production

The cytotoxicities of MPMs and RAW264.7 cells were decreased by the increment of concentration of competitive inhibitors, L-NMMA, NAME or arginase, and nitrite production by RAW264.7 cells was also decreased at high concentration of these chemicals in comparison with control group. The control group means MPMs activated with LPS alone or RAW264.7 cells with IFN-γ alone. Correlation coefficient between cytotoxicity to *T. vaginalis* and nitrite production by RAW264.7 cells was 0.655 ($p < 0.01$) (Fig. 2). LPS-activated MPMs did not produce appreciable amount of nitrite.

Addition of excess arginine (1 mM) to culture media containing L-NMMA, NAME, arginase restored the cytotoxic ability of activated macrophages to *T. vaginalis* (data not shown), confirming that an arginine-dependent mechanism was involved.

Effect of H_4B on the cytotoxicity to *T. vaginalis*

H_4B, cofactor of NO synthase, increased the cytotoxicity to *T. vaginalis* by MPM, not by RAW264.7 cells. H4B reduced the nitrite production by RAW264.7 cells on the contrary (Fig. 3). Nitrite productions by LPS-activated MPMs were minimal.

Effect of $FeSO_4$ on the cytotoxicity to *T. vaginalis* and nitrite production

Addition of $FeSO_4$ in the culture of MPM inhibited the cytotoxicity to *T. vaginalis*. RAW264.7 cells treated with $FeSO_4$ reduced the cytotoxicity to *T. vaginalis* and nitrite production (Fig. 4).

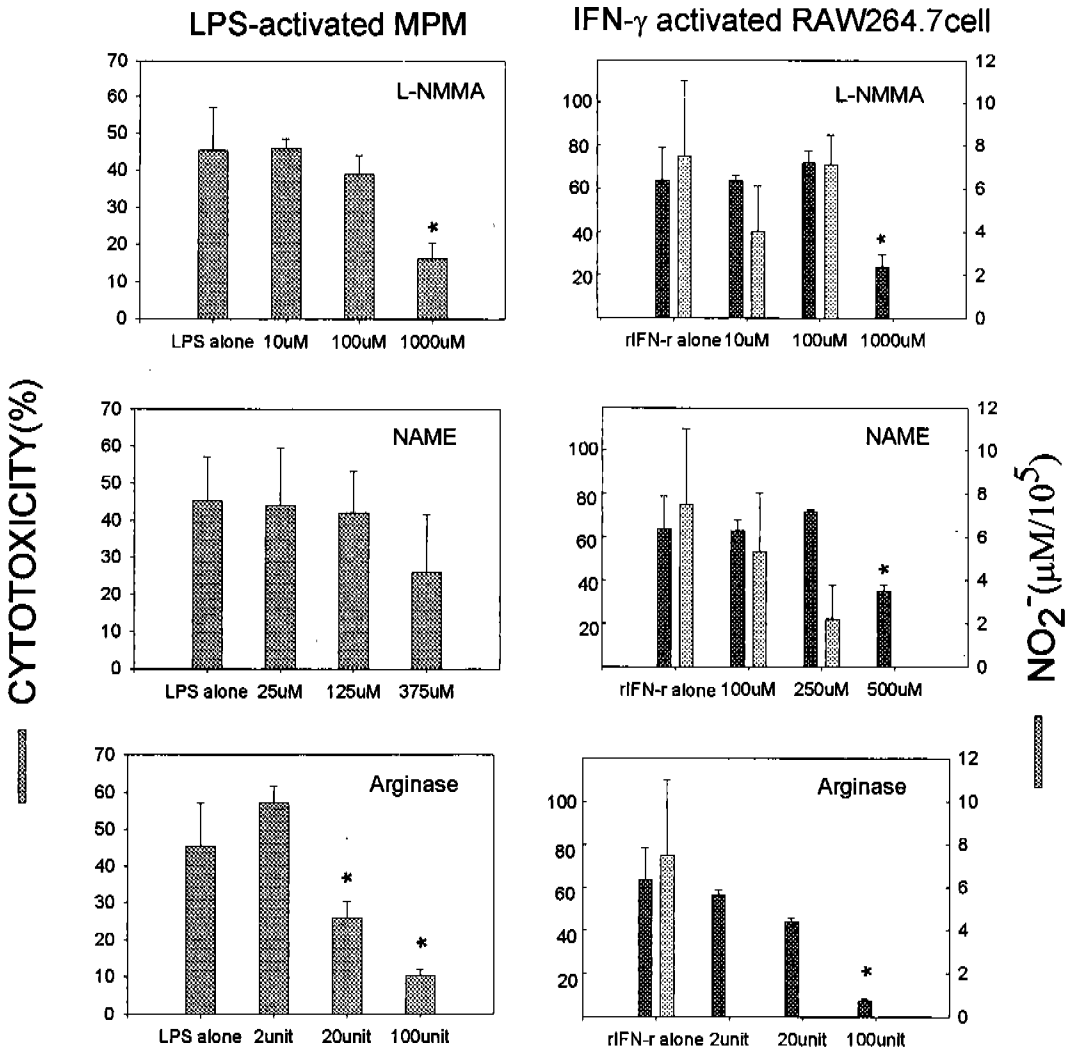

Fig. 2. Effects of L-NMMA, NAME, arginase on the cytotoxicity to *T. vaginalis* and nitrite production by LPS-activated mouse peritoneal macrophages (MPMs) and IFN-γ-activated RAW264.7 cells. Control macrophages are MPMs and RAW264.7 cells activated with LPS alone and IFN-γ alone, respectively. *$p < 0.05$, Mann-Whitney test

DISCUSSION

Nitric oxide (NO) is involved in diverse biological functions including regulation of vascular relaxation, long-term potentiation in neurons and the cytotoxic action of macrophages (Moncada *et al.*, 1991; Nathan, 1992).

Nitric oxide is derived from the guanidino nitrogen of L-arginine. The reaction is catalyzed by the cytosolic enzyme, NO synthase (NOS), of which there are at least two distinct types. One is constitutive in neuronal tissue and endothelium. The other (iNOS) is induced by IFN-γ, TNF-α and LPS in the endothelium, neutrophils, hepatocytes and macrophages (Cunha *et al.*, 1993).

L-NMMA is known to be a non-selective inhibitor of various NO synthase, and NAME inhibits constitutive NOS and is a much less effective inhibitor of the inducible isoform

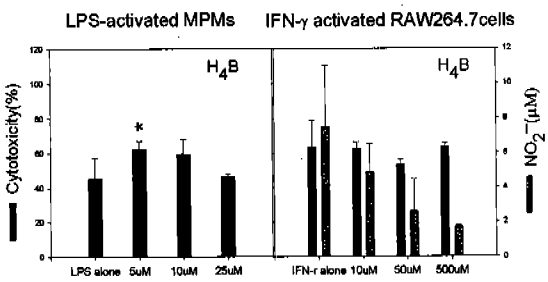

Fig. 3. Effect of H_4B on the cytotoxicity to *T. vaginalis* and nitrite production by LPS-activated mouse peritoneal macrophages (MPMs) and IFN-γ-activated RAW264.7 cells. Control macrophages are MPMs and RAW264.7 cells activated with LPS alone and IFN-γ alone, respectively. *p < 0.05, Mann-Whitney test

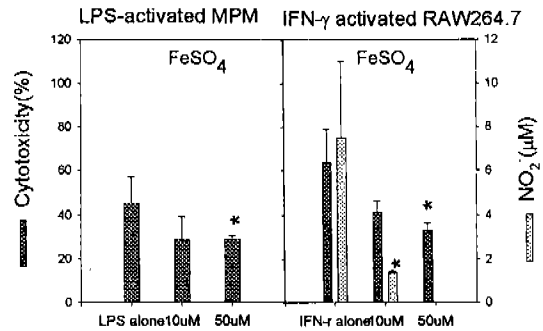

Fig. 4. Effect of $FeSO_4$ on the cytotoxicity to *T. vaginalis* and nitrite production by LPS-activated mouse peritoneal macrophages (MPMs) and IFN-γ-activated RAW264.7 cells. Control macrophages are MPMs and RAW264.7 cells activated with LPS alone and IFN-γ alone, respectively. *p < 0.05, Mann-Whitney test

(Knowles and Moncada, 1994). To observe the role of NO in cytotoxicity to *T. vaginalis* by macrophage, we use L-NMMA and NAME as arginine analogues. L-NMMA and NAME showed similar effects in reducing the cytotoxicity and nitrite release by MPMs and RAW264.7 cells at higher concentration of two arginine analogues, although each of L-arginine analogue may act on different type of NOS.

Arginase, an enzyme that competes with NO synthase for L-arginine by converting it to L-ornithine and urea, completely inhibited the nitrite production and decreased the cytotoxicity to *T. vaginalis* by RAW264.7 cells. The addition of excess L-arginine to macrophage culture medium containing L-NMMA, NAME or arginase completely restored the cytotoxicity. Decrease of both cytotoxicity to *T. vaginalis* and nitrite production by MPMs and RAW264.7 cells after treatment with arginase and NOS inhibitor suggest that cytotoxicity of macrophage against *T. vaginalis* involve arginine-dependent production of nitric oxide.

NO^+-containing chemicals such as sodium nitrite and sodium nitroprusside might partly exhibit the toxicity to *T. vaginalis* through inhibition of hydrogenosomal enzyme activity (Ryu and Lloyd, 1995; Ryu *et al.*, 1995). When sodium nitrite was added to *Entamoeba histolytica* and *Plasmodium falciparum in vitro*, NO is chemically generated from sodium nitrite at acid pH 5 and nitrogen derivatives might be toxic by themselves to protozoa (Rocket *et al.*, 1991; Lin and Chadee, 1992). Toxicity of NO appears to be due to the iron-scavenging properties of such compounds, resulting in iron depletion of the target cells and subsequent inactivation of iron-requiring molecules, such as aconitase (a Kreb s cycle enzyme) and iron-sulfur clusters of the mitochondrial respiratory chain (Hibbs *et al.*, 1988).

In this experiment, exogenous $FeSO_4$ decreased the cytotoxicity to *T. vaginalis*, an observation that is consistent with previous report of similar ferrous iron inhibition of macrophage cytotoxicity against various parasites such as *S. mansoni* (James and Glaven, 1989), *E. histolytica* (Lin and Chadee, 1992) and *Leishmania* (Mauel *et al.*, 1991). Probably excess iron might restore or protect iron-dependent enzyme of target cell. *T. vaginalis*.

NOS activity is dependent not only on the substrate, L-arginine, but also on the availability of reduced cofactors, such as tetrahydrobiopterin (H_4B). As our result shown, tetrahydrobiopterin (H_4B) increased the cytotoxicity to *T. vaginalis* by MPMs, an observation that is in accord with result of Mellouk *et al.* (1994) which H_4B enhanced antiplasmodial activity by human and murine hepatocyte. On the contrary, nitrite released by RAW264.7 cells was decreased with increment of H_4B concentration. For the

further study, H_4B biosynthesis inhibitor (2,4-diamino-6-hydroxypyrimidine) will be applied for macrophage culture to elucidate the role of H4B on the cytotoxicity to *T. vaginalis* and nitrite production by RAW 264.7 cells.

From above results, it is assumed that nitric oxide plays an important role in host defense mechanism of macrophages against *T. vaginalis*.

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=초록=

질편모충에 대한 대식세포의 세포독성에 있어서 NO의 역할

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활성화된 대식세포에서 생산되는 NO가 질편모충에 대해 세포독성이 있는지를 관찰하고자 질소 중간산물에 영향을 주는 약제를 첨가한 후 nitrite 생산 및 세포독성에 미치는 영향을 관찰하였다. 대식세포로는 마우스(BALB/c) 복강 대식세포와 마우스 복강 내 종양세포인 RAW264.7 세포로 LPS(lipopolysaccharide)나 rIFN-γ로 활성화시켜 사용하였다. 세포독성의 측정을 위해서 질편모충을 methyl-[^{3}H]-thymidine으로 표지하였고 NO의 측정은 Griess reagent를 사용하여 시행하였다. 마우스 복강 대식세포는 LPS로 활성화시켰을 때 질편모충에 대한 세포독성이 대조군에 비해 증가하였고, RAW264.7 세포는 rIFN-γ 또는 rIFN-γ 및 LPS로 활성화시켰을 때 대조군에 비해 세포독성 및 nitrite 생산량은 유의하게 증가하였다. LPS로 활성화시킨 마우스 복강 대식세포와 IFN-γ로 활성화시킨 RAW264.7 세포에 NO 생산에 영향을 주는 NG-monomethyl-L-arginine(L-NMMA), NG-nitro-L-arginine methyl ester(NAME), arginase를 첨가하였을 때 약제 농도를 증가시킴에 따라 질편모충에 대한 세포독성과 nitrite 생산이 감소하였다. NO synthase cofactor인 tetrahydrobiopterin(H_4B)을 마우스 복강 대식세포에 넣었을 때 질편모충에 대한 세포독성이 증가하였다. Ferrous sulfate를 두 종류의 활성화시킨 대식세포에 첨가하였을 때 질편모충에 대한 세포독성과 nitrite생산이 감소하였다. 이상의 성적을 종합하면 대식세포의 활성화에 따라 NO 생산 및 세포독성이 증가하였고, NO 생산을 저하시키는 약제들은 활성화된 복강 대식세포 및 RAW264.7 세포에 의한 질편모충에 대한 세포독성을 현저히 감소시키는 것으로 보아 NO는 질편모충에 대한 대식세포의 숙주 방어기전에서 중요한 역할을 감당할 것으로 생각된다.

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