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In vitro Induction Pattern of Nitrite, TNF-α and IFN-γ from Mouse Macrophage Activated with Trematodes Antigens

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흡충류 항원으로 감작한 마우스 대식세포에서의 Nitrite, TNF-α 및 IFN-γ 생성

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국문 초록

기생충감염시 cytokine으로 활성화된 대식세포가 방어기전의 effector cell로 작용할 때 분비하는 nitric oxide의 양 및 TNF-α와 IFN-γ의 분비정도와 nitric oxide와의 상관관계 등을 알아보기 위하여 3종의 흡충류, *Fasciola, Paragonimus, Schistosma*의 조항원(*100*㎎/ml)을 마우스 복강내에 주사한 후 24시간, 72시간, 9일간격으로 마우스의 대식세포(1×10^6/ml)를 분리하여 RPMI 배지(10% FCS 첨가)에서 48시간 배양후 nitrite, TNF-α 및 IFN-γ를 ELISA로 정량하여 다음과 같은 결과를 얻었다.

Nitrite 생성정도는 *Fasciola* 조항원으로 24시간 감작시킨 대식세포에서 가장 높게 나타났으며(140μM/ml) *Paragonimus* 항원군에서는 24시간에 최고치에 달하였다가(34uM/ml) 시간이 경과함에 따라 점차 감소하였다.

IFN-γ는 *Paragonimus* 항원군에서만 대조군에 비해 높았으며 9일 경에 최고치를 보였다(475ng/㎖). TNF-α는 *Schistosoma*항원군에서는 nitric oxide의 생성과 분비 양상이 일치하였다.

위의 결과에 의하면, 흡충류항원으로 감작된 마우스 대식세포의 nitric oxide 생성에 영향을 미치는 cytokine의 종류는 흡충류에 따라 차이를 보였으며, 이 중 *Paragonimus*항원에 의해서는 IFN-γ의 분비가 촉진되는 것으로 나타났고, *Schistosoma*의 경우에는 TNF-α가 nitric oxide의 생성에 관계함을 알 수 있었다.

Key words : *Nitrite*, TNF-α, IFN-γ, *Fasciola, Paragonimus, Schistosoma*

INTRODUCTION

Macrophages are activated for most cytotoxic effector activities by a two-stage reaction process known as priming and triggering.[14] Activated macrophages release many kinds of enzymes, cytokines, and reactive oxygen or nitrogen intermediate.[16, 19, 22]

Nitric oxide(NO) is a chemical messenger and appears to play an important function as an effector molecule of host resistance to a variety of pathogens.[13, 15]

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The expression and activity of NO are highly regulated by exocrine and autocrine signals.[1, 10] Inducible NO synthesis in macrophages is up-regulated primarily by the activation effects of Interferon gamma(IFN-γ).[3, 4] Tumor necrosis factor(TNF) has also been reported to generate nitric oxide synergistically with IFN-γ in *vitro* and *in vivo*.[2, 8, 9, 12, 21]

Recombinant TNF has reduced parasitemia of *Plasmodium yoeli* when injected with *in vivo*, but has not shown the same effect *in vitro*.[6] In case of *Schistosoma mansoni*, arginine-dependent, macrophage-mediated killing of schistosomula was observed.[7]

3 kinds of trematodes, *Fasciola* species, *Paragonimus* species and *Schistosoma japonicum* are very common throughout the world and severe endemic diseases according to geographical distribution. In this study, we questioned the relationship between NO production and IFN-γ and TNF-α in the 3 species of trematodes.

MATERIALS AND METHODS

Mice

Female balb/c mice 6-8 weeks old were purchased from the Chemical Research Institute of Korea(Tajeon, Korea).

Parasite antigens

Freeze-dried adult worms of *Fasciola* species, *Paragonimus* species and *Schistosoma japonicum* were homogenized in 0.01M phosphate buffer, pH 7.2 with a glass homogenizer at 4℃. The homogenates were centrifuged at 10,000g at 4℃ for 30min and the supernatant then stored at -70℃ until use. Protein estimation was done by the method of Lowry *et al*(1951).[11]

Mice injection and Macrophages harvest

Balb/c mice was injected with 3 kinds of Trematodes (*Fasciola* sp., *Paragonimus* sp. and *Schistosoma japonicum*) antigen(100μg/ml) into the peritoneal cavity. After 24, 72 hours and 9 days, mice were killed by cervical dislocation and residential macrophages were washed from the peritoneal cavity with RPMI 1640. After centrifugation at 170g for 10min at 4℃, the cell pellet was resuspended in complete media(RPMI 1640 containing 10% FCS). When erythrocytes were visible, the cell pellet was treated with 0.2% NaCl for 30s. Adherent macrophage monolayers were obtained by plating the cells in 6 well plastic trays(Corning, U.S.A.) at 1×10^6 cells/ml for 2 hours at 37℃ in 5% CO_2/95% air. Nonadherent cells were removed by suction and freshly prepared complete media were added. Culture supernatant was assayed for the production of IFN-γ, TNF-α and nitrite after 48 hours cultivation.

Measurement of nitrite production

Nitrite concentration in macrophage culture supernatant was assayed by a standard Griess reaction.[5] The Griess reagent consists of 1 part 0.1% naphthylenediamine dihydrochloride in distilled water plus 1 part 1% sulfanilamide in 5% concentrated H_3PO_4, the 2 parts being mixed together within 12 hours of use and kept chilled. A volume of 600μℓ of reagent was mixed with 100μℓ of supernatant and incubated 30 min in the dark. Absorbance of the chromophore formed was measured at 540nm. $NaNO_2$ was used as a standard.

Cytokines assay

Production level of IFN-γ and TNF-α was quantitated by means of sandwich ELISA(enzyme linked immunosorbent assay), using recombinant mouse TNF-α and IFN-γ(Genzyme), and a polyclonal rat anti-mouse TNF-α (Genzyme) and monoclonal anti-murine IFN-γ(Genzyme) as the conjugate.

RESULTS AND DISCUSSION

Groups of mice were injected with *Fasciola, Paragonimus* species and *Schistosoma* japonicum antigen 1ml

(100μg/mℓ) and sacrificed at 24, 72hours and 9 days time interval. Macrophages pooled from injected animals and non-injected controls were cultured for 48 hours and nitrite, IFN-γ and TNF-α production in the culture supernatants were quantitated by ELISA.

Table 1 shows that *Fasciola* antigen can induce nitrite effectively from the activated murine macrophages. Production level of nitrite decreased according to the time course. Macrophages harvested 1 day after antigen injection revealed the maximum value(140.0 μM/ml). As time goes by, however, the production level sharply decreased. Vincendeau and Daulouede(1991)[23] reported that macrophages from the 10th day of infection exerted antiproliferative effect on *Trypanosoma musculi* and this activity was maximum around 14th day of infection. At that time nitrite production paralleled development of macrophage trypanostatic activity. In our experiments nitrite production pattern in *Fasciola* antigen injection was different at all(Fig 1.). Many authors reported that *IFN*-γ and TNF-α secreted by macrophage and T cell are activating signals to macrophage cytotoxicity.[20] But the involved cytokine types were different from species to species, and these differences were also observed *in vitro* and *in vivo*.[6] In our results macrophage activated with *Fasciola* antigen secreted large amount of

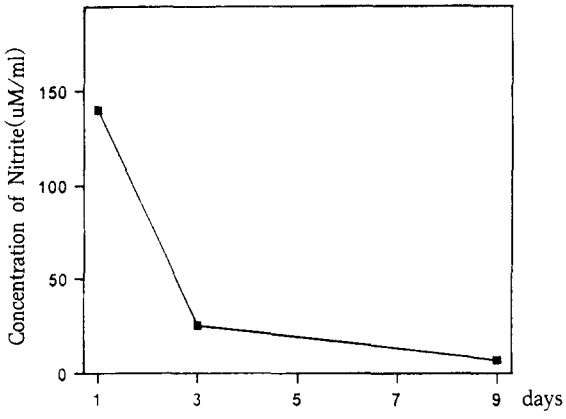

Fig. 1. Production pattern of Nitrite in *Fasciola* antigen injected mouse macrophages **(μM/ml)

NO at first time, but the amount markedly decresed at 9 days after injection. Parasite will have developed many methods for counteracting such host defense mechanisms. Parasite also may stimulate production of another kinds of cytokines that inhibit NO production.[18] Decrease of NO production in *Fasciola* antigen injection is speculated one of the escape mechanisms of parasite.[17]

When *Paragonimus* antigen was injected, nitrite secretion pattern had a tendency in accordance with that

Table 1. Nitrite and IFN-γ concentrations at three time intervals in ***Fasciola*** antigen injected mouse macrophages

	Fasciola (100μg/ml)		
	Time intervals(day)		
	1	3	9
Nitrite (μM/ml)	140.0±110.5	25.5±3.4	7.1±1.5
control	18.1±7.2	12.0±5.3	3.0±1.1
IFN-γ (ng/ml)	89.8±25.5	55.1±17.8	68.2±21.6
control	105.2±63.2	78.2±20.5	69.8±25.4

Results are expressed as mean±S.D. of triplicate samples.

of IFN-γ. As the amounts of IFN-γ increased, nitrite secretion was also augmented risen(Table 2 and Fig. 2). These results suggest the role of IFN-γ in nitrite production.

It was considered that TNF-α secretion level was related to the NO production, but IFN-γ was not detected. When *Schistosma* antigen was injected, NO production pattern was closely related the TNF-α. It was presumed that IFN-γ couldnot exert influence upon NO production pattern because IFN-γ level was below than control.

In case of *Schistosoma* species antigen, secretion pattern of nitrite and *TNF-α* coincided well(Table 3 and Fig. 3). one day after injection showed the highest value NO production. The amounts of secretion, however, sharply decreaed three days after injection, but nine day after injection, value increased again. These results are consistent with that of *Schistosoma mansoni* of James *et al.*[7] Accoding to Ohshima *et al.*,(1994)[17] animals sacrificed from 11 weeks to 13 weeks after infection excreted higher concentrations of nitrite. These results sug-

Table 2. Nitrite and IFN-γ concentrations at three time intervals in ***Paragonimus*** antigen injected mouse macrophages

	Paragonimus (100μg/ml)		
	Time intervals(day)		
	1	3	9
Nitrite (μM/ml)	23.4±2.7	26.0±3.3	60.2±20.0
control	18.1±7.2	12.0±5.3	3.0±1.1
IFN-γ (ng/ml)	95.5±34.7	425.6±52.7	475.8±108.1
control	105.2±63.2	78.2±20.5	69.8±25.4

Results are expressed as mean±S.D. of triplicate samples.

Table 3. Nitrite and IFN-γ concentrations at three time intervals in ***Schistosoma*** antigen injected mouse macrophages

	Schistosoma (100μg/ml)		
	Time intervals(day)		
	1	3	9
Nitrite (μM/ml)	34.1±9.5	15.9±7.2	22.3±3.0
control	18.1±7.2	12.0±5.3	3.0±1.1
TNF-α (ng/ml)	81.3±14.7	31.1±6.9	63.5±10.7
control	5.3±1.2	4.5±0.9	5.8±1.8
IFN-γ (ng/ml)	92.1±45.3	60.9±26.2	54.2±14.5
control	105.2±63.2	78.2±20.5	69.8±25.4

Results are expressed as mean±S.D. of triplicate samples.

gest that NO production persist long time in case of *Opisthorchis viverrini* and *Schistosoma* infection.

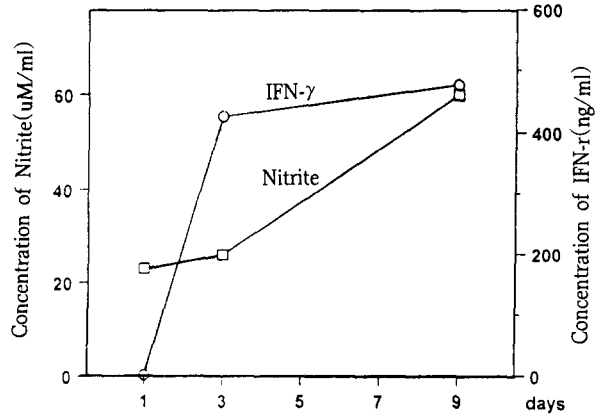

Fig. 2. Relationship of Nitrite an IFN-γ in *Paragonimus* antigen injected mouse macrophages

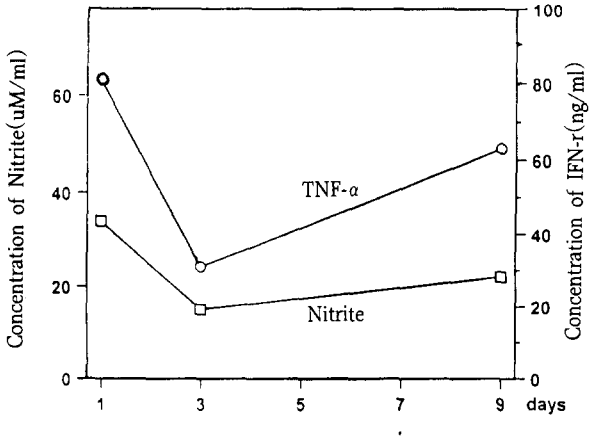

Fig. 3. Relationship of Nitrite and TNF-α in *Schistosoma* antigen injected mouse macrophages

CONCLUSION

Macropahge activated with parasite infection can be the effector cells of immune defense mechanism. To estimate the production levels of nitric oxide, TNF-α and IFN-γ and to study the relationship between nitric oxide and these two kinds of cytokines, 3 species of trematodes, *Fasciola* sp., *Paragonimus* sp., *Schistosoma japonicum* antigen(100μg/ml) were injected into the peritoneal cavity of the balb/c mice. Macrophage(1×10^6 cells/ml) was harvested from the injected mice after 24, 72hours, 9 days and cultured in RPMI 1640 media(10% FCS) for 48hours. The culture supernatants were quantified the concentration of nitrite, TNF-α and IFN-γ by ELISA.

The production level of nitrite revealed the highest (140μM/ml) value in the supernatant from the macrophage activated with *Fasciola* antigen for 24 hours and in 9 days supernatant(60μM/ml) of *Paragonimus* antigen group. Nitrite secretion arrived at the highest level (34mM/ml) within 24 hours and decreased in course of time in case of *Schistosoma*. IFN-γ induction level was higher than that of control only in *Paragonimus* group, and showed the highest production after 9 days sensitization. TNF-α production accorded with NO secretion pattern in *Schistosoma* case only.

These data suggest that a type of cytokine exerted influence upon the production level of NO from macrophage depends on the species of trematode antigens. And *Paragonimus* antigen was considered to facilitate the secretion of IFN-γ. It seemed that NO synthesis in *Schistosoma* case was related to TNF-α release.

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