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Cellular mechanisms of nonspecific immunity to intracellular infection: cytokine-induced synthesis of toxic nitrogen oxides from L-arginine by macrophages and hepatocytes

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1. Summary

Nitric oxide (NO) produced by cytokine-treated macrophages and hepatocytes plays a vital role in protective host responses to infectious pathogens. NO inhibits iron-sulfur-dependent enzymes involved in cellular respiration, energy production, and reproduction. Synthesis of L-arginine-derived nitrite (NO_2^-), the oxidative end product of NO, directly correlates with intracellular killing of *Leishmania major*, an obligate intracellular protozoan parasite of macrophages: the level of NO_2^- production is a quantitative index for macrophage activation. The competitive inhibitor of NO synthesis, monomethylarginine (N^GMMLA), inhibits both parasite killing and NO_2^- production. For *Leishmania*, the parasite itself participates in the regulation of this toxic effector mechanism. This participation is mediated by parasite induction of tumor necrosis factor α (TNFα), an autocrine factor of macrophages: NO synthesis by interferon-γ (IFN-γ)-treated cells can be blocked by monoclonal antibodies to TNFα. NO production by IFNγ-treated hepatocytes is of special interest in malaria infections: sporozoite-infected hepatocytes kill the intracellular malaria parasite after treatment with IFNγ; this killing is inhibited by N^GMMLA.

Key words: Leishmania; Malaria; Nitric oxide; Macrophage; Hepatocyte; Interferon-γ; Tumor necrosis factor α

2. Introduction

Intracellular infectious pathogens, sequestered and protected from the extracellular environment, are notoriously difficult to eradicate by humoral host defenses and drug therapy. Malaria schizonts and leishmania amastigotes are unrelated intracellular pathogens that successfully evade host resistance, complete their life cycle, and induce disease. Once transmitted from insect vector to vertebrate host, both parasites invade their preferred target cells: malaria sporozoites penetrate hepatocytes and mature into liver-stage schizonts; amastigotes infect and replicate exclusively in the phagolysosomes of macrophages. It is at these stages of the parasite life cycle that the massive amplification in numbers of infectious microorganisms occurs with coincident increase in disease potential.

Current efficacy of vaccination and chemotherapy to augment body defense during these disease stages falls far short of success. Antibodies directed against the circumsporozoite protein only rarely have been shown to completely inhibit sporozoite penetration of primary human hepatocyte cultures [1]. Once inside the liver cell, sporozoites are likely protected from further antibody attack (S. Mellouk et al., unpublished results). Design of effective drug therapy is confounded by issues of toxicity and of achieving adequate drug levels not only in tissues, but also inside the infected cell. Elimination of parasites and resolution of disease can only occur by changes in the intracellular milieu of the infected cell itself from one that is supportive of parasite replica-

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tion, to one that is hostile to survival.

Recent studies document IFNγ and TNFα as important endogenous signals that induce such changes and activate cellular antimicrobial activities [2]. However, the proximate effector molecule(s) that cytokine stimulated cells actually employ to eliminate infectious pathogens remained a mystery. We show in this report that nitrogen oxidation of L-arginine is required for such microbicidal activity. The intermediate product of this reaction, NO, is an essential mediator for intracellular destruction of leishmania amastigotes by cytokine-activated macrophages [3], and its production is achieved and regulated through a complex interaction of host target cell, cytokine signals, and the pathogen itself. We further show that this mechanism is involved in the protection of IFNγ-treated hepatocyte against sporozoite in vitro. Both observations document synthesis of NO as a major host defense reaction against intracellular parasites.

3. Results and Discussion

3.1. *Nitrogen oxidation of L-arginine by cytokine-activated cells: a cellular mechanism for antimicrobial activity*

Mammals synthesize both nitrite and nitrate (NO_3^-) as a consequence of acute inflammation. Details of the cellular biochemical pathways that produce of NO_2^- and NO_3^- by nitrogen oxidation are incompletely defined, but the basic pathways are known [4, 5]. The terminal guanido nitrogen of L-arginine is oxidized, and released as an unstable highly reactive intermediate, NO. Citrulline is formed as a byproduct, and NO, with a half-life of milliseconds, is further oxidized to NO_2^- and NO_3^-. The labile NO intermediate is highly toxic. NO toxicity is mediated through inactivation of iron-dependent enzyme systems [6, 7]. NO inhibits oxidoreductases of the mitochondrial electron transport chain, the Krebs' citric acid cycle enzyme, aconitase, and still other enzymes necessary for DNA replication [8–10]. Each of these enzymes contains a catalytically active iron molecule linked to a sulfur group that is degraded by NO and released as a iron-nitrosyl complex [11].

3.2. *Intracellular destruction of* L. major *by activated macrophages*

We and others have demonstrated the importance of IFNγ in the resolution of leishmanial disease [12]. To examine whether IFNγ-treated macrophages use NO as an effector molecule in the destruction of intracellular amastigotes, we used a specific inhibitor

TABLE 1

Effects of N^GMMLA[a] and arginine depletion[b] on microbicidal activity by IFNγ-treated macrophages.

Cells incubated 72 h in:	% Infected macrophages (mean ± SEM)	% Microbicidal activity[c]
Addition of excess L-arginine		
Medium	53 ± 6	0
+ 0.1 mM N^GMMLA	58 ± 4	0
20 U/ml IFNγ	3 ± 1	94
20 U/ml IFNγ		
+ 0.1 mM N^GMMLA	35 ± 8	34
+ 0.1 mM N^GMMLA + 1.4 mM L-arginine	8 ± 3	84
Addition of arginase		
Medium	65 ± 8	0
+ 5 U/ml arginase	62 ± 6	5
20 U/ml IFNγ	10 ± 5	85
+ 5 U/ml arginase	43 ± 34	34

[a] L-arginine was added to cultured medium at the same time that *L. major*-infected macrophages were treated with IFNγ and N^GMMLA.
[b] Arginase was added to culture medium 1 h before addition of amastigotes and IFNγ. [c] % Microbicidal activity was calculated by determining the change in % infected macrophages in treated cultures compared to the medium-treated controls [3].

of nitrogen oxidation of L-arginine, N^GMMLA [13]. Macrophages treated with 10 U/ml IFNγ develop potent intracellular killing capability: >80% of macrophages infected with amastigotes kill and eliminate the intracellular parasites within 72 h. Microbicidal activity in replicate cultures treated with N^GMMLA was completely blocked at concentrations >0.1 mM [3].

Two different methods were used to confirm the role of L-arginine-derived NO as an effector molecule for microbicidal activity: (a) the specificity of N^GMMLA inhibition was documented by adding excess L-arginine to the reaction mixture, and (b) L-arginine was depleted in the culture medium by adding arginase (Table 1). Both approaches confirmed the essential role of L-arginine in expression of microbicidal activity. Further, macrophage cytotoxicity against *Leishmania* amastigotes directly correlated with the metabolism of L-arginine to NO_2^-, the oxidative and stable end product of the short-lived NO intermediate (Table 2). Thus, the levels of NO_2^- in the culture medium were quantitative indices of macrophage activation and cytotoxic effector function.

3.3. *Role of the parasite in generation of NO by cytokine-activated target cells*

Like other macrophage effector activities, the initiation of nitrogen oxidation requires multiple signals. Such signals are provided by both endogenous (cytokines) and exogenous (bacterial cell wall components, endotoxic lipopolysaccharides) stimuli [2]. In uninfected cultures, macrophages treated with IFNγ and bacterial endotoxic lipopolysaccharides (LPS) produce 60 ± 5 μM NO_2^-/72 h, but cells treated with IFNγ alone produce only 2 ± 2 μM. The level of nitrogen oxidation achieved after exposure of uninfected macrophages to IFNγ alone is not sufficient to affect the viability of intracellular amastigotes: quantitative dose-response analysis of NO_2^- levels and cytotoxicity show that at least 30 μM NO_2^-/72 h are required for cytotoxicity against this intracellular parasite. But macrophages infected with leishmania and then treated with IFNγ *alone* kill the intracellular parasites and produce high levels (>60 μM NO_2^-/72 h) of NO_2^-. These results suggest that the *Leishmania* parasite itself provides a second signal to IFNγ-activated macrophages that induces both NO and microbicidal activity. This amastigote-associated activation signal was independent of LPS: (a) amastigotes structurally lack lipid A and other LPS components; (b) polymixin B, an antibiotic that competitively inhibits binding of lipid A to cell membranes, did not affect induction of NO and macrophage cytotoxic activity by amastigotes; (c) the ability of amastigotes to induce NO and macrophage cytotoxic activity was heat-labile, while that of LPS heat-stable; and (d) culture medium from amastigote-infected macrophages were negative for LPS (<5 pg/ml LPS) as

TABLE 2

Correlation between NO_2^- production and microbicidal activity by IFNγ-treated macrophages[a].

Cells treated 72 h with:	% Microdicidal activity	NO_2^- (μM/10^6 cells/72 h)
Medium	0	1 ± 2
10 U/ml IFNγ	90	65 ± 11
+ 1 mM N^GMMLA	4	1 ± 0
+ 5 U/ml arginase	30	8 ± 5

[a] Culture fluids were assayed for NO_2^- by the Greiss colormetric reaction at 543 nm absorbance. Cell smears were examined by light microscopy for determination of microbicidal activity [3].

TABLE 3

Effects of anti-TNFα on microbicidal activity and NO_2^- production[a].

Culture conditions	% Microbicidal activity	NO_2^- (μM/10^6 cells/72 h)
Macrophages		0
+ amastigotes		0
+ amastigotes + IFNγ	97 ± 6	40 ± 9
+ anti-TNFα	3 ± 3	7 ± 4
+ anti-TNFα + (TNFα at 3 h)[b]	87 ± 4	41 ± 9
+ control antibody	91 ± 0	42 ± 5

[a] Resident peritoneal macrophages were treated with 50 μg/ml anti-TNFα or an identical isotype control antibody, followed by the addition of amastigotes and 10 U/ml IFNγ. After 72 h, microbicidal activity and NO_2^- production were measured [3].
[b] 100 U/ml TNFα was added 3 h after the addition of the anti-TNFα.

measured by limulus ameobocyte lysate assay.

In a survey of several different cytokines in combination with IFNγ, only TNFα induced nitrogen oxidation of L-arginine in treated macrophages [2, 14]. To test whether this TNFα pathway was involved in amastigote induction of NO_2^- production, we infected macrophages in the presence of anti-TNFα antibodies and then treated these infected cells with IFNγ. Anti-TNFα antibody, but not an irrelevant control antibody, totally abrogated NO_2^- production and microbicidal activity (Table 3). The anti-TNFα inhibition of both activities was restored by addition of excess TNFα. Thus, amastigotes trigger the synthesis and release of TNFα, which then acts as an autocrine signal to induce NO-dependent microbicidal activity in the IFNγ-activated macrophages.

3.4. *Hepatocyte NO as an effector molecule for IFNγ-induced killing of exoerythrocytic stages of* Plasmodium berghei

There are several reports that document a role for IFNγ in the control of malaria sporozoite infection in hepatocytes [15, 16]. The effector mechanisms that destroy the sporozoites in such models are not yet known. A recent report demonstrated that conditioned medium from LPS-treated Kupffer cells induce production of NO in hepatocyte cultures [17]. This observation suggested to us that this toxic mediator may play a role in resistance to malaria. To confirm this hypothesis, we examined whether N^GMMLA could affect hepatocyte destruction of sporozoites in vitro (Table 4). *P. berghei*-infected hepatocyte cultures treated with IFNγ show a significant decrease in number of intracellular parasites (schizonts). This IFNγ-induced protection was inhibited by the addition of N^GMMLA (S. Mellouk et al., submitted). These preliminary data suggest that NO may effect the IFNγ-induced killing of this protozoan parasite by the hepatocyte, a cell not normally involved in immune reactions. In many respects, the malaria sporozoites in hepatocyte cultures closely parallel those with leishmania amastigotes in macrophages. It is quite likely that NO production by the hepatocyte and the macrophage are regulated by similar TNFα-dependent mechanisms. However, the cellular sources and signals for induction of TNFα production in our studies are not yet identified.

TABLE 4

Effects of N^GMMLA on killing of *P. berghei* schizonts by IFNγ-treated hepatocytes[a].

Culture conditions	Schizonts/10^5 hepatocytes	% Inhibition of schizont development
Hepatocytes exposed to sporozoites and treated with:		
Medium (control)	1770	0
0.5 mM N^GMMLA	1790	0
1000 U/ml IFNγ	710	60
1000 U/ml IFNγ + 0.5 mM N^GMMLA	1280	27

[a] Primary hepatocyte cultures from BALB/c mice were exposed to *P. berghei* sporozoites and treated 3 h after infection with IFNγ and N^GMMLA. After 48 h, % inhibition was calculated by comparing the number of schizonts in the experimental cultures with that in control [1, 15].

Although hepatocytes comprise >99% of cells in our cultures, it is possible that a few stromal fibroblasts, endothelial cells, or Kupffer cells provide TNFα or TNFα-inducing cytokines. Indeed, previous studies show that conditioned-medium from LPS-stimulated Kupffer cells induces NO in hepatocytes [17].

4. Conclusions

Complex multi-signal control of macrophage activation has been studied for a number of years. The activation process is tightly regulated for the expression and local release of highly toxic effector molecules. Indeed, the NO that is generated by macrophages to kill tumor cells and microorganisms also has deleterious effects on the effector cell that generated the NO. Restriction of NO production to cells that have actually encountered a pathogen makes teleological sense: only cells that have a pathogen to kill will generate the NO; the amount of toxic NO leakage into surrounding normal tissues will thereby be minimized. For the *Leishmania*, the pathogen itself provides an activation signal. Other intracellular parasites that grow in macrophages, such as metacyclic *L. major* promastigotes, *Toxoplasma gondii* [18], and *Francisella tularensis* are each susceptible to the lethal effects of NO, and are each able to trigger NO_2^- synthesis in macrophages treated solely with IFNγ. The nature of the activation signals

provided by such intracellular microorganisms is a fascinating question. In the case of Gram-negative bacteria, LPS is the most likely candidate. For other non-bacterial parasites, the signal is unknown, but appears from our studies with *L. major* to be independent of LPS.

NO is produced not only by cells of the immune system, but also by a variety of other cells types including hepatocytes and endothelial cells. It is possible to envision the upregulation of NO synthesis as an antidote to pathogenic agents sequestered in all sorts of host cells throughout the body. Recognition of NO as a common effector molecule for destruction of diverse pathogens is but the first step in a series of studies whose aim is the nonspecific protection from infectious agents. The design or identification of drugs that can selectively regulate this effector molecule could prove useful in infectious disease.

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