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Cytokines and nitric oxide as effector molecules against parasitic infections

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SUMMARY

Nitric oxide (NO) derived from L-arginine by the catalytic action of inducible NO synthase (iNOS) plays an important role in killing parasites. Many cell types express high levels of iNOS when activated by a number of immunological stimuli which include interferon-gamma (IFN-γ), tumour necrosis factor alpha, and lipopolysaccharide. IFN-γ is typically produced by the Th1 subset of CD4+ T cells, whose differentiation depends on interleukin-12 (IL-12) produced by macrophages. Mice with a disrupted iNOS gene were highly susceptible to *Leishmania major* infection compared with similarly infected control wild-type mice. The mutant mice developed significantly higher levels of TH1-cell response compared with the control mice, suggesting that NO is likely to be the effector molecule in the immunological control of this and other intracellular parasitic infections. To ensure their survival, the *Leishmania* parasites have evolved effective means to inhibit NO synthesis. The highly conserved major surface glycolipids, glycoinositol-phospholipids and lipophosphoglycan (LPG), of *Leishmania* are potent inhibitors of NO synthesis. Furthermore, LPG can also inhibit IL-12 synthesis, thereby indirectly blocking the induction of iNOS. The evolutionary and therapeutic implications of these findings are discussed.

1. INTRODUCTION

There is considerable current interest in nitric oxide (NO) which plays an important role in a variety of biological functions (Moncada & Higgs 1993; Liew & Cox 1991; Nathan & Xie 1994; Bredt & Snyder 1994; Marletta 1994; Hibbs *et al*. 1990). These include platelet aggregation, neurotransmission and cytotoxicity. NO is derived from L-arginine together with molecular oxygen in a reaction catalysed by the enzyme NO synthase (NOS), with NADPH and tetrahydrobiopterin as cofactors. NO is very unstable with a half-life between 3 and 15 s, and it is usually measured as its oxidative products, nitrite and nitrate, in culture supernatants or serum. NOS can be competitively inhibited by L-arginine analogues such as L-N^G-monomethyl arginine (L-NMMA).

NO is derived from the guanidino nitrogen of L-arginine. L-NMMA has a methyl group at the guanidino nitrogen, therefore preventing the utilization of this nitrogen for NO synthesis. The inhibition is highly specific in that the D-enantiomer, D-NMMA, is completely inert. The availability of NOS specific inhibitors such as L-NMMA has contributed greatly to study in the NO field, helping it to become one of the most exciting and prolific areas of biomedical research.

There are three isoforms of NOS, the neuronal form (nNOS), the endothelial form (eNOS), and the inducible form (iNOS). They share a number of common structural features such as NADPH-, FAD-, FMN- and calmodulin-binding domains, but otherwise have little amino acid sequence homology. These are relatively large molecules, with molecular weights of 150 kDa for nNOS, and 130 for eNOS and iNOS. Furthermore, homodimerization is required for full biological activity. So far, the 3D structure of NOS is not available, nor is the precise L-arginine binding site known.

In response to acetylcholine and influx of calcium, eNOS will produce physiological amounts of NO which diffuse out of the endothelium and lead to vascular and muscular relaxation. nNOS also produces small amounts of NO in the neuronal cells in response to calcium and glutamate, and this is involved in glial cell activation and long term potentiation. iNOS is expressed in a variety of cell types, including macrophages, neutrophils and fibroblasts in response to a range of immunological stimuli, such as cytokines and lipopolysaccharide (LPS). Once induced, iNOS produces large amounts of NO mediating microbicidal and tumoricidal activities. It can also cause a range of immunopathologies.

2. ANTI-LEISHMANIAL ACTIVITY OF NO

In 1990, we demonstrated that macrophage killing of the leishmania parasite *in vivo* is mediated by NO from L-arginine (Liew *et al*. 1990). This was carried out by injecting infected mice with L-NMMA. Since then, a

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variety of micro-organisms have been shown to be sensitive to NO. These include *Cryptococcus neoformans*, *Schistosoma mansoni*, *Leishmania* spp., *Toxoplasma gondii*, *Plasmodium* spp., and *Trypanosoma* spp. (table 1). All these experiments were carried out in murine models using NOS inhibitors. Because the inhibitors are not NOS isoform specific, there has been controversy and uncertainty over some of the studies. To obtain a more definitive result, a strain of iNOS-deficient mice was constructed (Wei *et al.* 1995). Peritoneal macrophages from the heterozygous mice produce large amounts of NO when stimulated with LPS and IFN-γ *in vitro*. The cells from the mutant mice failed to do so.

In vitro, macrophages from the heterozygous or wild-type mice were very efficient in killing the leishmania parasites following activation by IFN-γ and LPS. In contrast, similarly stimulated cells from the mutant mice were unable to kill the parasites. The wild-type and heterozygous mice were able to control the infection by *Leishmania major*. In contrast, the mutant mice were uniformly highly susceptible to the infection (figure 1). This is so, despite the fact that the mutant mice developed a stronger Th1-cell response compared with the healing heterozygous or wild-type mice, producing more IFN-γ and less IL-4 in response to antigens or mitogens *in vitro*. These results provide compelling evidence that NO is an important effector molecule for the killing of intracellular parasites such as *Leishmania*.

The following scheme is now generally agreed to be valid. When *Leishmania* parasites, in the form of the flagellated promastigotes, infect an individual, they go straight into the macrophages. Here, depending on the genetic constitution of the host, which is yet to be defined, there can be preferential induction of IL-12, which drives the development of Th1 cells. The Th1 cells produce IFN-γ which activates macrophages to produce NO, and this kills the parasites. In contrast, in susceptible individuals, there is preferential induction of IL-4 which drives the differentiation of Th2 cells which produce more IL-4 that can inhibit the activation of macrophages and the synthesis of NO. This is certainly not the only control mechanism in leishmaniasis. However, it is likely to be one of the major events during cutaneous leishmania infection, making it arguably the best example of the polarized function of Th1 and Th2 cells, and hence an almost perfect biologically-relevant model for the study of immune regulation.

Table 1. *Micro-organisms susceptible to NO*

	restriction by NO		
	in vitro	*in vivo*	references
intracellular micro-organisms[a]			
Rickettsia conorii	+	+	(Feng & Walker 1993; Turco & Winkler 1993; Feng *et al.* 1994)
Cryptosporidium parvum	+	+	(Leitch & He 1994)
Trypanosoma cruzi	+	+	(Gazzelini *et al.* 1992*a*; Muñoz-Fernández *et al.* 1992; Vespa *et al.* 1994)
Plasmodium falciparum	+	—	(Rockett *et al.* 1991)
P. berghei	+	—	(Nüssler *et al.* 1993)
P. yoeli yoeli	+	—	(Nüssler *et al.* 1991)
P. chabaudi chabaudi	—	+	(Taylor-Robinson *et al.* 1993)
Mycobacterium leprae	+	—	(Adams *et al.* 1991)
M. tuberculosis	+	+	(Chan *et al.* 1992; Chan *et al.* 1995)
Legionella pneumophila	+	—	(Summersgill *et al.* 1992)
Toxoplasma gondii	+	—	(Adams *et al.* 1990)
Listeria monocytogenes	+	+	(Beckerman *et al.* 1993; Boockvar *et al.* 1994; MacMicking *et al.* 1995)
Leishmania major	+	+	(Green *et al.* 1990; Liew *et al.* 1990; Wei *et al.* 1995)
Mycobacterium bovis	+	—	(Flesch & Kaufmann 1991)
Chlamydia trachomatis	+	—	(Mayer *et al.* 1993)
Entamoeba histolytica	+	—	(Lin & Chadee 1992)
Candida albicans	+	+	(Vasquez-Torres *et al.* 1995)
Cryptococcus neoformans	+	—	(Granger *et al.* 1988; Lee *et al.* 1994)
Francisella tularensis	+	+	(Anthony *et al.* 1992; Fortier *et al.* 1992; Green *et al.* 1993)
extracellular micro-organisms			
Staphylococcus aureus	+	—	(Malawista *et al.* 1992; Kaplan *et al.* 1996)
Borrelia burgdorferi	+	—	(Modolell *et al.* 1994)
Entamoeba histolytica	+	—	(Lin & Chadee 1992)
Naegleria fowleri	+	—	(Fischer-Stenger & Marcianocabral 1992)
Trypanosoma musculi	+	—	(Vincendeau & Daulouede 1991)
Schistosoma mansoni	+	—	(James & Glaven 1989)

[a]Faculative and obligate intracellular micro-organisms.

3. HOW LEISHMANIA PARASITES EVADE THE HOST'S KILLING MECHANISM

Parasites are evolutionarily highly successful organisms, and they must have developed highly sophisticated mechanisms to combat the host's killing mechanisms. For example, *Leishmania* can inhibit the synthesis of NO directly, and block the development of Th1 cells indirectly via the inhibition of IL-12 synthesis.

There are two highly conserved major surface molecules on the *Leishmania* parasites. These are glycoinositolphospholipids (GIPLs) and lipophosphoglycan (LPG). There are about 20 million such molecules of GIPLs on the promastigotes and this number remains more or less constant at the amastigote stage. LPG is a large molecule displaying various lengths of sugar chain. Five million molecules of LPG are present on the promastigotes' surface. LPG is essential for the differentiation of the parasite in the insect

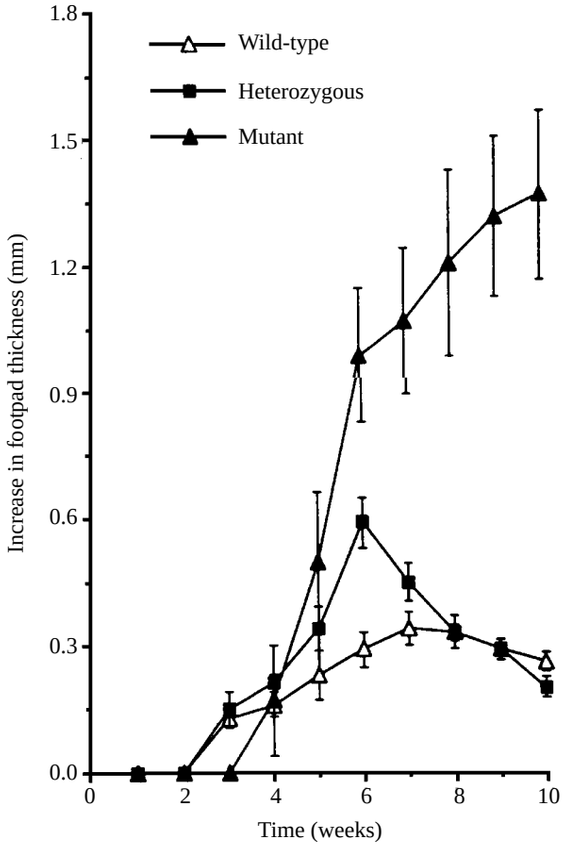

Figure 1. Mice lacking iNOS failed to control *Leishmania major* infection. Groups of five wild-type, heterozygous and mutant mice were infected in the footpads with 1×10^6 stationary phase *L. major* (LV39) promastigotes. Lesion development was measured at regular intervals with a dial calliper and expressed as footpad thickness increase. The wild-type and heterozygous mice achieved spontaneous healing, but mutant (iNOS-deficient) mice failed to do so. (Data from Wei *et al.* 1995.)

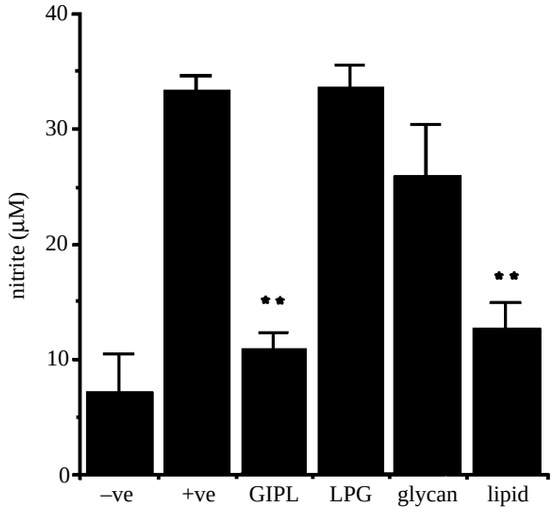

Figure 2. The inhibitory activity of the GIPLs is in the lipid fraction. The murine macrophage cell line, J774, was incubated with medium alone (−ve), or stimulated with 40 U ml^{-1} IFN-γ and 10 ng ml^{-1} of LPS (+ve), or pre-incubated with GIPLs (50 μM), LPG (50 μM) or the lipid and glycan fractions isolated from GIPLs (100 μM) for 4 h before stimulation. Nitrite concentrations in the culture supernatants were determined by the Griess Method. (Data are from Proudfoot *et al.* 1995.)

gut. However, very few of these molecules are present at the mammalian amastigote stage. It is thought that the parasite sheds them within hours of entering macrophages. The reason for this is currently unknown.

We have recently shown (Proudfoot *et al.* 1995) that GIPLs markedly inhibit NO synthesis by macrophages stimulated with IFN-γ and LPS. The inhibition was dose- but not time-dependent, and was effective before or even after macrophage activation. The inhibition of NO synthesis paralleled the replication of the parasites. All the inhibitory activity is contained in the lipid fraction (figure 2).

LPG consists of a monoalkyl lysophosphatidyl inositol anchor, a hexosaccharide core, a polymer of repeating phosphodisaccharides of galactose and mannose, and a neutral mannose cap. There are some species-specific differences in the carbohydrate side-chains of the helical phosphodisaccharide repeats. *L. mexicana*, *L donovani* and *L. major* release hydrophilic phosphoglycan (PG), and PG epitopes are also found on some surface proteins such as secreted acid phosphatase from *L. mexicana*. LPG and related molecules may interact directly with carbohydrate binding sites of macrophage receptors or indirectly with the complement receptors CR1 and CR3. LPG can be broken down to various pieces by phosphoinositol phospholipase C digestion and mild acid hydrolysis. Neither LPG nor any of its fractions can induce NO synthesis on their own. In contrast, LPG, especially PG, induces large amounts of NO when added together with, or soon after, the stimulation of IFN-γ. The individual repeats, and the lipid core are not effective. The effect of PG on NO synthesis is highly time- and dose-dependent. Addition of PG with IFN-γ leads to the induction of NO, whereas pre-treating the cells with PG overnight results in a dose-dependent inhibition of NO synthesis (figure 3) (Proudfoot *et al.* 1996). A number of PG repeats have been synthesized. These ranged from a single Gal-Man-phosphate to multiple repeats all with a decenyl lipid tail. All the fragments were able to synergize with IFN-γ to induce NO synthesis, with the longer repeats being more effective. In contrast, all the fragments were able to inhibit NO synthesis when they were added before the cells were activated with IFN-γ and PG. The shorter fragments were more effective as inhibitors than the longer fragments. The inhibition of NO synthesis is at the transcriptional level of iNOS expression, as shown by Northern blot analysis, and did not involve the inhibition of TNFα. Thus, LPG of *Leishmania* can strongly regulate the expression of iNOS and the leishmanicidal activity of murine macrophages.

It is tempting to speculate that the evolutionary significance of GIPLs and LPG may be as follows: GIPLs inhibit the induction of NO synthesis by macrophages,

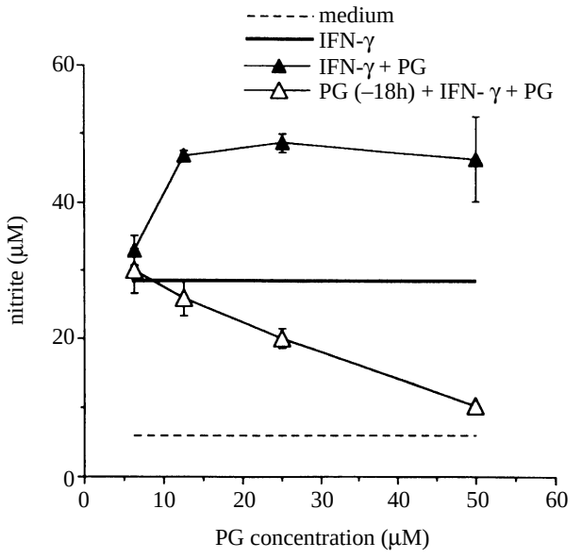

Figure 3. The synthesis of NO by macrophages in response to *L. major* PG is time- and dose-dependent. J774 cells were cultured with medium alone, IFN-γ (40 U ml^{-1}) alone, or *L. major* PG at graded concentrations added 18 h before or at the same time as IFN-γ (40 U ml^{-1}). Cells pre-incubated with PG at −18 h were washed and restimulated with IFN-γ + PG at the same time as the untreated cells. Supernatants were removed for nitrite determination 24 h after IFN-γ treatment. Data are mean ± s.e.m., $n = 6$. (Data are from Proudfoot *et al*. 1996.)

thereby contributing to the survival of the parasites. The host counters this by making use of LPG to enhance NO synthesis. The parasite's answer is to shed its LPG as soon as it enters the macrophage, or in the case of naive hosts, to use the PG to inhibit subsequent NO synthesis by the host.

Leishmania parasites can also use LPG to inhibit the induction of IL-12. IL-12 is a major inducer of the Th1 cells which produce IFN-γ, the substance which activates macrophages to produce NO. When murine macrophages were infected with amastigotes, they produced large amounts of IL-12. In contrast, when the cells were infected with promastigotes, no IL-12 production was detectable. GIPLs but not LPG could synergize with IFN-γ to induce IL-12 synthesis. Furthermore, synthetic PG was able to inhibit the induction of NO synthesis in murine macrophages by IFN-γ and LPS in a dose-dependent manner.

4. CONCLUSIONS

The 'macrophage→IL-12→IFN-γ→NO→leishmanicide' circuit is essentially complete. *Leishmania* parasites possess a number of survival mechanisms, one of which is the switching off of the NO production machinery. GIPLs and LPG can both inhibit NO synthesis by IFN-γ-activated macrophages, most effectively in non-immune hosts (prior to exposure to IFN-γ). LPG can also inhibit the induction of IL-12 production, thereby indirectly, perhaps most effectively, subverting the production of NO, and hence the survival of the parasites. In this context, IL-12 and NO on the one hand, and GIPLs and LPG on the other, are important molecules for the intriguing interaction between host and parasite. Whether this is a general principle, applicable to other pathogenic infections remains to be explored.

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