Role of Nitric Oxide in Parasitic Infections

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INTRODUCTION

Nitric oxide (or nitrogen monoxide), a labile and highly reactive gas, was until relatively recently considered primarily a member of a family of undesirable environmental pollutants and potential carcinogens. Interest in the physiological function of this molecule was potentiated by its identification as the endothelium-derived relaxing factor mediating vascular smooth muscle relaxation and vasodilation (139) and as a neuronal messenger molecule (47). Since then, it has been further identified as a major regulatory molecule of the immune system and a principal cytotoxic mediator of activated immune effector cells. In light of the significant role it plays as a messenger molecule and regulator of cellular functions, nitric oxide was named “molecule of the year” in 1992 (32).

Formation of NO has now been demonstrated by a variety of cells and tissues, including vascular endothelial cells, neurons, platelets, neutrophils, adrenal cells, respiratory epithelial cells, fibroblasts, muscle cells, and hepatocytes, as well as activated macrophages and macrophage-like cells (12, 130). As a cytotoxic/cytostatic effector molecule, NO has been shown to inhibit the growth and function of a diverse array of infectious disease agents, including various bacteria and fungi, as well as protozoan and helminthic parasites (200). The full range of its influence on parasitic infections, however, probably extends beyond its direct role in protective immunity to encompass its other physiologic effects as well. Thus, a better understanding of the function of NO and its derivatives in the protective mechanisms of the host and the parasitic mechanisms of the pathogen may reveal much about the delicate balance of the host-parasite relationship.

NITRIC OXIDE SYNTHESIS

The biochemical pathway of NO synthesis in mammalian cells was initially elucidated in activated murine macrophages, in which it was found that NO production resulted from the enzymatic oxidative deamination of L-arginine to produce L-citrulline (reviewed in reference 200). Experiments with L-[guanidino-15N2]arginine in the culture media revealed that NO is formed from one of the chemically equivalent guanidino nitrogens (67). NO undergoes oxidative degradation in aqueous solution to form nitrite (NO−2) and nitrate (NO3−) (67). Activated mouse macrophages produce nitrite and nitrate in culture, and in vivo injection of bacterial lipopolysaccharide (LPS) results in increased nitrate concentration in blood as well as nitrate excretion in the urine (182). Several assays are
available to detect the relatively high level of NO production generated by activated macrophages: NO$_2^-$ can be measured by a simple colorimetric assay known as the Greiss reaction (58). NO$_3^-$ can be likewise detected as NO$_2^-$ after reduction by bacterial nitrate reductase or cadmium (54, 68), and citrulline can also be detected in a colorimetric assay (98).

The conversion of arginine to citrulline and NO is catalyzed by the enzyme NO synthase (NOS), of which three different isoforms, representing distinct gene products, have been identified (for reviews, see references 107, 127, and 130). In endothelial cells and neurons, the constitutive low-output form of NOS (cNOS) produces small amounts of NO for short periods. This form is activated by calcium binding to the enzyme cofactor calmodulin (17, 34). Neuronal NOS was the first of these enzymes to be cloned (16). The molecule demonstrates binding sites for NADPH, flavin adenine dinucleotide, and flavin mononucleotide, which support the oxidation of a guaianidino nitrogen of arginine, as well as calmodulin. Tetrahydrobiop-terin is also required as a cofactor for enzymatic activity. The macrophage NOS gene is shorter than the neuronal NOS gene, but the molecules share about 50% identity in amino acid sequence (76, 92, 106, 108, 202), including the binding sites for multiple cofactors (107).

Macrophage NOS has been reported to contain a tightly bound calmodulin molecule which maintains an active conformation (24), and thus, in contrast to cNOS, regulation of the activity of the macrophage form of the enzyme is not at the level of Ca$^{2+}$ binding. This form of NOS is not constitutively expressed. Enhanced transcription of the gene for this enzyme, termed the inducible NOS (iNOS), is observed in response to stimulation with certain cytokines or LPS (39, 43). Transcriptional control of iNOS occurs via a complex structure of promoter and enhancer elements; induction by LPS involves binding of nuclear factor NF-κB heterodimers p50/c-Rel and p50/RelA at an NF-κB site in the promoter, while gamma interferon (IFN-γ) action involves binding of interferon regulatory factor 1 to an interferon regulatory factor-binding site (111). In addition to macrophages, many other cell types, including fibroblasts (197), hepatocytes (131), muscle cells (84), astrocytes (96), and endothelial cells (which contain both the cNOS and iNOS forms) (83, 107), possess the iNOS enzyme. The levels of NO produced by the iNOS enzyme are far higher than those produced by cNOS, and this is therefore referred to as the high-output pathway of NO synthesis.

Both cNOS and NOS are initially produced as inactive monomers. The NOS molecule has a heme-binding site in its N-terminal domain and contains iron-protoporphyrin IX. Upon reduction and treatment with carbon monoxide, it shows optical properties similar to those of cytochrome P-450 (reviewed in references 110 and 177). Moreover, NOS has significant homology to mammalian cytochrome P-450 reductase in the C-terminal region (16). Dimerization of NOS leads to approximation of the C- and N-terminal domains in a trans arrangement, allowing the reduction of the Fe(III) moiety required for binding molecular oxygen (110, 177). In the case of cNOS enzymes, dimerization follows binding of l-arginine, tetrahydrobiopterin, and heme. Binding of calmodulin that has been activated by high levels of Ca$^{2+}$ induces a further conformational change in the dimer, resulting in enzymatic activity. As mentioned above, in the case of iNOS, calmodulin binds to the molecule even in the presence of only trace amounts of Ca$^{2+}$. Dimerization and enzymatic activity occur following the binding of tetrahydrobiop-terin, l-arginine, and heme (reviewed in reference 127). As currently understood, the mechanism of action of NOS involves an initial hydroxylation of l-arginine to N$^\omega$-hydroxy-arginine followed by an additional oxidation in which heme ferric peroxide nucleophile is a critical reaction intermediate (110, 177).

The function of all forms of NOS can be inhibited by N-substituted arginine analogs such as N$^\omega$-monomethyl-l-argi-nine (NMMA), aminoguanidine, or N-nitroarginine methyl-ester. The availability of such inhibitors allows assessment of the effects of the arginine-NO pathway in vivo (54). In culture, NO synthesis is also inhibited by the activity of the arginine enzyme, which utilizes arginine to form ornithine and urea, thus depleting the substrate for NO. The potential for differential control of these two enzymes, perhaps even within the same cell type, as a mechanism of in vivo regulation of NO production could have an important influence on the outcome of the inflammatory process (28). Flavoprotein binders or calmodulin inhibitors can also inhibit the activity of NOS (181). Finally, it has been shown that NOS is inhibited by NO itself, through a direct effect on enzyme-bound heme (62).

**NITRIC OXIDE ACTION**

Owing to its small size and exceptional membrane permeability, it seems obvious in retrospect that NO would be well suited to serve as a chemical messenger in cell-cell interactions. The biological effects of NO may actually involve several related forms, however, including not only free-radical nitric oxide but also nitrosonium cation and nitroxylanions (177). Biologically important reactions include those with transition metal ions and redox forms of oxygen, as well as thiols and possibly other nucleophilic centers (reviewed in references 175 and 177).

As mentioned previously, NO or NO derivatives are involved in intercellular communications regulating blood vessel dilation and post-synaptic-presynaptic neuronal interaction (34). The function of the relatively small amount of NO generated through the low-output neuronal and endothelial cNOS mechanisms appears to center around its ability to stimulate the enzyme guanylate cyclase to produce elevated levels of cyclic GMP (cGMP), probably through NO binding of heme iron in the active site of the latter enzyme (70, 109). Intracellular cGMP then serves as a second messenger in the activation of protein kinases.

From the perspective of its function in parasitic infections, more interest has been generated by the participation of the high-output iNOS pathway of NO production in cell-mediated cytotoxicity and host immunity (Fig. 1). The biochemical basis of this function appears to be primarily due to the inactivation of critical metabolic pathways (200). Through the formation of iron-dinitrosyl-dithiolate complexes, NO inactivates several key metabolic enzymes with [4Fe-4S] prosthetic groups at their catalytic sites (94, 200). This has been associated with loss of intracellular iron by the target cell (66). Nitrosylation of Fe-S centers is associated with inactivation of the aconitase enzyme of the Krebs cycle (41, 177), NADPH:ubiquinone oxidoreductase and succinate:ubiquinone oxidoreductase of the electron transport chain (41, 55, 177), and ribonucleotide reductase, which is involved in DNA synthesis and cell proliferation (91). It should be noted that although these are the major targets of NO activity identified in mammalian cell targets, other potential enzymatic targets for NO may exist in parasites. For example, leishmania parasites utilize an iron-containing superoxide dismutase (118), and inactivation of this enzyme could considerably weaken the ability of the parasites to withstand attack by reactive oxygen intermediates.

In addition to reaction with transition metals, thiols, and amines (133, 177), NO can react with superoxide anions to form peroxynitrite anions, which rapidly decay to release the...
highly reactive hydroxyl radicals (10). Production of NO appears to be independent of the respiratory burst, which normally produces reactive oxygen species including superoxide in phagocytic leukocytes (71). In a mixed inflammatory response, however, the potential exists for production of both reactive nitrogen and oxygen species. Interestingly, NOS itself also possesses the capacity to generate reactive oxygen species under conditions of low arginine (145).

The half-life of NO is a matter of seconds in oxygenated, aqueous solutions, and for that reason it has been assumed that its effects could be delivered only to a target located very close to the NO source. This makes good sense from the point of view of the host, to avoid toxicity to surrounding tissues. Indeed, production of NO can result in enzymatic inhibition within the cellular source itself (42, 62), and evidence is accumulating that NO contributes to some pathologic conditions (86). The possibility exists, however, that certain metalloproteins serve as carriers for NO, thus extending the range of its activity. For example, Fe(III) heme proteins bind NO with much lower affinity than do Fe(II) heme proteins and are able to release NO readily (177). An intriguing example of the biologic activity of such a carrier is provided by the recently discovered salivary heme protein of *Rhodnius prolixus*, the reduviid bug vector of the protozoal parasite *Trypanosoma cruzi*, which reversibly binds NO (150). This molecule is capable of dissociating NO to the host tissues while the insect is taking a blood meal, and it thus serves to facilitate vasodilation and improve feeding. In addition, proteins with S-nitrosylated thiol groups have been shown to activate guanylate cyclase, and it has therefore been suggested that S-nitrosoproteins may serve as stable carriers capable of mediating certain physiologic activities of NO (176).

**REGULATION OF NO PRODUCTION**

**Cytokine Signals Enhancing NO Production**

The best-studied example of cytokine-induced NO production almost certainly involves the murine macrophage. In any discussion of cytokine signals leading to NO production, it is important to remember that not all macrophages are equivalent and, consequently, that signalling requirements may differ on the basis of the tissue source and differentiation state (bone marrow, monocyte, peritoneal cavity, etc.), recruitment stimulation (relatively quiescent resident cells versus inflammatory cells elicited by mild or strong irritants), and genetic background (e.g., mouse strain) of the cells that were utilized. Within that context, however, it has been widely accepted for several years that to become fully cytotoxic, the macrophage must receive two consecutive signals (31, 64). For most biological targets, activation for full cytotoxicity correlates with enhanced expression of the iNOS gene and secretion of NO (39, 43).

In the early 1980s, it was established that IFN-γ is the most common primary signal for macrophage activation (138, 152, 184). These early studies generally employed low concentrations of bacterial LPS to provide the second, or “triggering,” signal for activation (64). The sequence of exposure to these two types of signals is evidently critical to the activation process, with simultaneous or sequential exposure to IFN-γ and LPS required to induce NO synthesis (105). It has subsequently been demonstrated that tumor necrosis factor alpha (TNF-α) provides the major physiological second signal for IFN-γ-primed macrophages (39, 43). TNF-α production is induced endogenously in macrophages by LPS as well as other bacterial components, and recent evidence indicates that parasite components may likewise provide a direct stimulus for TNF-α production (59). Peritoneal macrophages elicited by thioglycolate broth, which are the most biochemically active type of inflammatory but noncytotoxic macrophage, are stimulated by IFN-γ exposure alone to endogenously produce the TNF-α second signal for full cytotoxic activation and NO production (137). Other cytokines, notably interleukin-2 (IL-2) (29), may also serve an accessory function with IFN-γ for macrophage activation. In addition, there have been occasional reports of other cytokines participating in activation (reviewed in reference 31), but these cases may be peculiar to the cell type or target organism.

The demonstration that several nonmacrophage cells also possess the iNOS pathway has made it clear that cells outside the traditional immune network can likewise play a role in protective immunity. These cell types also require stimulation by multiple cytokine signals for activation. In general, these
activation signals may be provided by combinations of cytokines including IFN-γ, TNF-α, and IL-1, as well as LPS (83, 130, 131, 134, 144, 200).

Table 1. Some parasite defense mechanisms against NO

| Inhibition of: | Through induction of: |
|----------------|-----------------------|
| Th1-related IFN-γ production | IL-4, IL-10, PGE₂, NO (negative effects on antigen presentation and cell proliferation) |
| IFN-γ-mediated NO production | IL-10, TGF-β, IL-4(±), IL-13(±), NO (inactivation of NOS) |
| NO antiparasitic effects | Conversion to nonsusceptible forms (e.g., anaerobic) |

*PGE₂, prostaglandin E₂, ± indicates that these cytokines have also been shown to be stimulatory under certain conditions.

**Signals Down-Regulating NO Production**

Since many products of activated effector cells, including NO, can be harmful to the host, mechanisms exist to inhibit their production or restrict their toxic effects (Table 1). Several cytokines are known to suppress proliferation or production of IFN-γ by Th1 lymphocytes, thus limiting effector cell activation and indirectly inhibiting NO production. Some of these, such as IL-4 and IL-10, are produced by the Th2 subset of lymphocytes, in addition to other cell types, and contribute to the functional dichotomy of cellular immune response which appears to play a role in the progression of many parasitic diseases (119, 163). Production of NO can also be regulated more directly at the level of the effector cell by certain cytokines, which may reduce the transcription of mRNA for iNOS or affect its stability (for a review, see reference 13). Thus, IL-4 and IL-10, as well as transforming growth factor β (TGF-β) and a cytokine known as macrophage-deactivating factor, have been shown in many systems to directly modulate NO production. The extent of their effects, however, varies dramatically under various experimental conditions. Their inhibitory function is extremely dependent on the exposure of macrophages to these cytokines before introduction of the activating stimuli (38, 51, 101). The ability of IL-4, IL-10, and TGF-β to inhibit macrophage cytotoxic activity is synergistic, so that exposure to a combination of these cytokines at concentrations that are ineffective individually results in significant suppression of NO production and cytotoxicity (136). Curiously, however, there have been a few reports that under certain conditions, IL-4 can activate macrophages for enhanced cytotoxicity (30, 178); the factors contributing to this alternative activity for IL-4 remain poorly understood. Another cytokine with apparent dual effects is IL-13, which has been shown to decrease NO production by LPS-stimulated granulocyte-macrophage colony-stimulating factor-derived bone marrow macrophages but to enhance NO production by LPS-stimulated macrophage colony-stimulating factor-derived bone marrow macrophages (40).

NO itself may ultimately regulate its own production; such autoinhibition could serve to limit excessive production and resultant tissue damage. As previously mentioned, NO can directly inactivate NOS (62). Another apparent mechanism involves direct inhibition by NO of Ia expression on IFN-γ-activated macrophages, which would limit antigen-presenting capability (169). Other studies also suggest that NO production by antigen-presenting cells blocks T-cell proliferation (157).

It should be kept in mind that physiological mediators other than cytokines may also regulate NO production. For example, prostaglandin E₂ is another macrophage product that has long been recognized for its regulatory activity. This may be due primarily to an effect on Th1 cells, resulting in decreased IL-2 and IFN-γ production (142); there have, however, been scattered reports that prostaglandins down-regulate NO production by activated macrophages (5, 146). In addition, recent reports indicate that the vasoactive peptide angiotensin II decreases iNOS expression in astroglial cells (21).

**Antiparasitic Effects of NO**

**Macrophage Activity against Parasite Targets**

**Schistosomiasis.** While the antimicrobial effects of NO have now been documented for a vast array of target organisms (200), one of the earliest demonstrations of its microbicidal activity was a study of the mechanism of macrophage cytotoxicity toward the larvae of a helminth parasite, *Schistosoma mansoni* (74). This complex multicellular trematode begins its life cycle within the mammalian host when the infective free-living cercarial stage penetrates the skin. It then undergoes a remarkable transformation into a physiologically adapted larval form known as the schistosomulum. These schistosomula rapidly leave the skin and migrate via the blood and lymphatics to the lungs, through which they transit over a period of days. Eventually, their journey through the bloodstream leads them to the liver, where male and female parasites mature further and mate. Adult worm pairs live for years within the mesenteric veins, where they produce hundreds of eggs daily. The immune response to eggs that become lodged in host tissue is responsible for the pathology of the disease schistosomiasis (15). Thus, the parasite remains extracellular throughout its interaction with the host, and it was observed several years ago that cytokine-activated macrophages were capable of killing the larval schistosomula by a mechanism that closely resembled that of macrophage cytotoxicity for other extracellular targets, i.e., tumor cells (44, 75, 172).

Activation of inflammatory peritoneal macrophages by IFN-γ and other cytokine cofactors or LPS induces the arginine-dependent production of NO, which serves as the effector molecule of parasite killing (74). Products of the macrophage respiratory burst are not required (161). Larval killing is inhibited by addition of either the arginine analog NMMA or excess iron to the culture media (74). More recently, an in vitro NO-generating compound has been shown to duplicate the effects of lymphokine-activated macrophages in vitro (135).

The principal targets of NO in the schistosome appear to be enzymes containing a catalytically active Fe-S group. Ultrastructural studies of skin-stage schistosomula cocultured with activated macrophages showed that unlike antibody-dependent killing, macrophage-mediated cytotoxicity is not directed against the parasite surface. Within the first hour of incubation, perturbation of the subtegumental muscle cells and mitochondria was noted. Progressive disintegration of parasite internal structures followed, such that by 48 h of incubation, the organisms were reduced to an intact tegumental "shell" surrounding severely vacuolated and disorganized internal tissues (114). This pattern of target damage previously observed in ultrastructural studies is consistent with the possibility that inhibition of larval metabolism is involved in macrophage-mediated parasite killing. Recently, we have found that inactivation of the aconitase enzyme or of the electron transport chain involved in mitochondrial respiration by various chemi-
clearcell-rich inflammatory reaction histologically reminiscent of the fate of challenge parasites in vaccinated mice indicated within the lungs and eventually disappeared (82). Investigation of challenge infection of previously vaccinated animals revealed that the lymph nodes draining the lungs (141, 174). In autoradiographic alveolar lavage produced IFN-γ from the lungs of vaccinated and challenged mice by bronchino-philic reaction in the lungs, while the macrophage phagocytes in resistance to Schistosoma infection. This hypothesis was investigated in a series of in vivo studies of protective immunity. In a murine model in which a high level of resistance against challenge S. mansoni infection is elicited by a single prior exposure to radiation-attenuated parasites, treatment with antibodies against CD4 or IFN-γ depleted immunity while treatment with antibodies against the Th2 cytokines IL-4 and IL-5 failed to diminish resistance (164, 173).

Several lines of evidence suggest that lymphokine-activated macrophages play a key role in this model. CD4+ T cells isolated from the spleens of vaccinated mice proliferated and produced substantial amounts of IFN-γ upon in vitro stimulation with parasite antigens (75, 191). That such a response also occurs in vivo is indicated by the observation that activated macrophages, displaying cytotoxicity for schistosomula as well as tumor cell targets, were recovered from the peritoneal cavities of vaccinated animals within 24 h after intraperitoneal injection of soluble parasite antigens (75). Genetic studies provided direct support for the proposed role of IFN-γ-activated macrophages in resistance to S. mansoni in vivo. In a series of experiments involving genetic crosses between high- and low-responder mouse strains, the ability to develop resistance segregated with the ability to develop activated larvicidal macrophages (reviewed in reference 72).

How might cell-mediated immunity participate in resistance to challenge schistosome infection? In vivo studies have shown that mice vaccinated with irradiated cercariae develop a massive increase in numbers of TA , oxidatively reactive macrophages in the lungs within 2 to 3 weeks (117). Challenge infection of immunized animals stimulated an anamnestic T-lymphocytic response in the lungs, while the macrophage population remained elevated (4). Lymphocytes recovered from the lungs of vaccinated and challenged mice by bronchoalveolar lavage produced IFN-γ, as did cells harvested from the lymph nodes draining the lungs (141, 174). In autoradiographic tracking experiments, most of the parasites from challenge infection of previously vaccinated animals were retained within the lungs and eventually disappeared (82). Investigation of the fate of challenge parasites in vaccinated mice indicated that larvae transiting the lungs became trapped in a mononuclear cell-rich inflammatory reaction histologically reminiscent of the delayed hypersensitivity response, a Th1-associated phenomenon (199).

Elimination in the lungs is a protracted process, lasting several weeks (82). We have recently found that late-lung-stage larvae are susceptible to killing in vitro by activated effector cells through the arginine-dependent pathway, as well as by exogenous NO (46, 135). Examination of the levels of cytokine mRNA in the lungs of vaccinated mice at the time during which attrition of challenge parasites is manifest showed abundant production of Th1-type cytokines, including IFN-γ, TNF, and IL-2, which are known to activate NO-producing effector cells (201). Moreover, the identification of high iNOS mRNA levels in the lungs at this time and the demonstration of the enzyme in inflammatory foci around the challenge parasites (201) give further credence to the possibility that the NO-mediated effector mechanism is operative in vivo. Finally, this conclusion is supported by the observation that treatment of vaccinated animals with the NO inhibitor aminoguanidine resulted in a markedly decreased level of resistance to challenge infection (201).

Leishmania. Aside from the helminth S. mansoni, the best examples of NO antiparasitic activity all involve protozoal targets. Of these, perhaps the most extensively studied parasites are those of the genus Leishmania, various species of which cause pathologic manifestations ranging from localized cutaneous lesions to lethal visceralizing systemic disease (61). There is abundant evidence in murine model systems that Th1-directed cell-mediated immune mechanisms are protective against leishmanial infection in vivo (reviewed in references 65 and 163). In a susceptible mouse strain, protection against Leishmania major infection can be obtained by transfer of Th1 cells or by immunization in such a way as to induce Th1 responses including IFN-γ production, e.g., with IL-12 as adjuvant (2). Leishmaniases are obligate intracellular parasites of macrophages, and the protective mechanism in this system ultimately involves cytokine activation of macrophages (or macrophage-related cells, e.g., Kupffer cells in the liver) to kill or inhibit the growth of these intracellular targets. A number of reports have shown that in vitro cytotoxicity against the intracellular form of leishmanias is mediated by NO (60, 61, 103, 112). In addition, treatment of L. major-infected mice with the NOS inhibitor NMMA resulted in substantially increased parasite loads and development of larger skin lesions (102, 200), validating the importance of this mechanism in regulating parasite growth in vivo. Experiments involving immunohistochemical staining with antibodies against iNOS, as well as quantitation of mRNA levels, revealed that the enzyme was expressed earlier in cutaneous lesions and draining lymph nodes of a strain of mouse that is resistant to L. major infection than in those of a susceptible strain. Macrophages were identified as the iNOS-producing cells within the skin lesions, and regions where iNOS levels were high contained few or no parasites (179). Macrophage production of NO and leishmanicidal activity were found in one study to be inhibited by the hydrogen peroxide inhibitor catalase, and it was concluded that hydrogen peroxide affects the levels of the cofactor tetrahydrobiopterin necessary for NOS function (99). Recently, mutant mice were produced through the disruption of the iNOS gene (195). Macrophages from these animals failed to produce NO after stimulation with IFN-γ plus LPS and likewise failed to control L. major infection in vivo, in what is perhaps the ultimate demonstration of the relevance of this effector mechanism (195).

Interestingly, direct interaction with L. major parasites appears to provide an adequate triggering stimulus for IFN-γ-stimulated resident peritoneal macrophages to become activated for production of NO, through the induction of TNF-α production by these cells (59). Such endogenous TNF-α serves as an autocrine stimulus for full activation of the IFN-γ-primed cells. Heat-killed parasites or other phagocytizable particles...
failed to provide an equivalent stimulus for TNF-α production in these studies (59), suggesting that the process of cell infection by the living parasite provides a unique activation signal. While interaction with a specific macrophage membrane receptor is an attractive hypothesis, the complete triggering process remains to be clarified. An unusual alternative mechanism, whereby membrane-bound TNF on CD47’ Th1 cells was able to activate macrophages alone or in combination with IFN-γ for leishmanicidal activity and NO production, has also been reported (11).

The ability of different inbred strains of mice to limit the growth of Leishmania donovani, as well as Mycobacterium species and Salmonella typhimurium, is controlled at a locus known as Lsh/Bcg/Ity, whose action is manifested principally at the level of the macrophage (204). A candidate gene has recently been cloned and designated Nramp (for natural resistance-associated macrophage protein) (151). Lsh/Bcg/Ity has pleiotropic effects, and the mechanism by which it controls the growth of these unrelated microorganisms remains controversial (204). The currently favored hypothesis involves Nramp regulation of early events in transmembrane signalling for macrophage priming/activation, via the eNOS-dependent activation of guanylate cyclase and the cGMP-dependent kinase pathway, which ultimately culminates in the TNF-α-dependent production of microbicidal levels of NO (151). These studies involve the use of bone marrow-derived macrophages, however, and it should be cautioned that others have observed no differences in NO production by IFN-γ- plus–LPS-stimulated inflammatory peritoneal macrophages from mice bearing the susceptible or resistant form of the gene (204).

The understanding that IFN-γ plays an important role in host defense against leishmanial infection has led to several clinical trials of its efficacy in the treatment of cutaneous or visceral disease (123). Most of these studies have involved evaluation of adjunct therapy with recombinant IFN-γ in combination with standard drug treatment with pentavalent antimonials, with the rationale that drug and activated effector cells would function synergistically to kill the parasite. While some of these trials have shown promise, the clinical utility of combination immunotherapy remains to be clarified.

In several systems, susceptibility to leishmanial infection can be correlated with the production of down-regulatory cytokines. As mentioned previously, prior treatment of macrophages with IL-4 inhibits IFN-γ-induced leishmanicidal activity and NO production in vitro, through an effect unrelated to the level of IFN-γ binding to the cells (100). Thus, in this system, Th2-related cytokines may inhibit protective immunity both at the afferent level (through decreased induction of Th1 response and IFN-γ production) and at the efferent level (through decreased activation by IFN-γ). Prior or simultaneous exposure to TGF-β likewise suppressed intracellular killing of leishmanias as well as NO production by lymphokine-activated murine peritoneal macrophages but had no effect on the ability of the cells to resist initial invasion by the parasite (128). Leishmania amazonensis infection has been shown to induce the endogenous production of TGF-β by murine peritoneal macrophages, and local production of TGF-β has been noted at the infection site in susceptible mice (7). Furthermore, injection of recombinant TGF-β into the infection site enhanced parasite replication in vivo, whereas injection of neutralizing anti-TGF-β antibody arrested lesion development (7), suggesting that induction of a regulatory cytokine by this parasite contributes to its escape from the development of protective immunity in the susceptible host. That this mechanism may be utilized by other parasite species is suggested by the finding that more cells staining positive for TGF-β production were observed in skin lesions of L. major-infected mice of a susceptible strain than in those of a resistant strain, whereas no strain-related differences were observed for IFN-γ or IL-4 production (179). Addition of exogenous IL-10 virtually eliminated killing of L. major by IFN-γ- or IL-7-treated murine peritoneal or bone marrow-derived macrophages in vitro provided that it was present during the process of macrophage activation (190). Perhaps more pertinent to the in vivo situation, however, was the observation that addition of neutralizing anti-IL-10 antibody or an IL-10-specific antisense phosphorothioate DNA-oligonucleotide led to enhanced killing by activated macrophages in the absence of exogenous IL-10, suggesting that IL-10 produced endogenously by the cells limits their function in an autocrine fashion (190).

Toxoplasmosis. The coccidian parasite Toxoplasma gondii is capable of invading virtually any nucleated cell type, causing significant damage to the central nervous system in individuals with lowered immune function (9, 167). Cell-mediated immunity, involving IFN-γ-activated macrophage effector cells, has been shown to play a role in resistance to this parasite (reviewed in reference 9). In T. gondii infection, IFN-γ may come not only from stimulation of CD45’ and CD85’ lymphocytes but also from natural killer cells (48, 166). Among the effector mechanisms contributing to macrophage-mediated antiparasitic activity is the production of NO (1). The toxoplasmastatic activity of IFN-γ-activated macrophages was dependent on the production of TNF-α by these cells, which appeared to function in an autocrine fashion to induce NO production (95). In this system, as opposed to the case of leishmania infection, triggering for NO production by IFN-γ-treated macrophages was independent of direct parasite-macrophage interaction, since it could be induced prior to infection (95). These investigators found good correlation between toxoplasmastatic activity and release of NO in vitro when they treated the macrophages with IFN-γ plus NMMA or an NADPH inhibitor during the 24-h period prior to infection but observed no correlation when the inhibitors were present only during the infection. They hypothesized, therefore, that reactive nitrogen intermediates do not directly affect parasite proliferation but, rather, exert their effects through activation of another effector mechanism. Interestingly, another group has reported the need for addition of exogenous TNF-α to IFN-γ-treated macrophages to achieve full activation for antitoxoplastic activity (168). These differing observations exemplify the need to take all experimental parameters into consideration when drawing conclusions about a biological activity. While both studies utilized resident murine peritoneal macrophages, among the factors that could be responsible for the discrepancy between the results in these studies is the use of cells from different mouse strains.

T. gondii has profound effects upon the central nervous system, as noted above. Microglial cells are the functional correlates of macrophages in the central nervous system, and recent studies indicate that they may function to inhibit parasite growth in this physiologic compartment. Thus, murine microglial cells that had been stimulated with IFN-γ and LPS inhibited T. gondii replication in vitro, and this activity correlated with induction of NOS activity and production of reactive nitrogen intermediates by these cells (22, 77). TNF-α was also seen to potentiate the effects of IFN-γ in this system (77). Electron-microscopic analysis of the cultures showed degenerative tachyzoites of T. gondii in the activated microglia (222). The antitoxoplasmal activity of IFN-γ-plus–LPS-activated microglia was blocked by neutralizing antibodies against TNF-α or by treatment with drugs known to inhibit TNF-α production (pentoxifylline and dexamethasone), indicating that, as in
other systems, the endogenous production of this cytokine provides the ultimate triggering signal (23). Surprisingly, however, antibodies against TGF-β1 also blocked the antiparasitic effect of these cells (23), suggesting an unusual protective role for this cytokine in this system.

**Trypanosomiasis.** The South American and African forms of trypanosomiasis are very different diseases. South American trypanosomiasis, or Chagas’ disease, is caused by the protozoan parasite *Trypanosoma cruzi* and in its chronic stages can result in myocardopathy and enlargement of the hollow viscera (mesoepithelium, megacolon). *T. cruzi* is an obligate intracellular parasite and lives in a variety of host cell types, especially muscle cells and macrophages (89). It has long been established that activated macrophages can kill *T. cruzi* in vitro (69). A series of reports have documented that IFN-γ and, under certain conditions, granulocyte-macrophage colony-stimulating factor stimulate macrophage trypanocidal activity (53, 148, 149, 198). Treatment of mice with neutralizing anti-IFN-γ antibody prior to and during infection with *T. cruzi* resulted in an increased parasite burden in both the blood and tissues and considerably shortened the survival time, demonstrating that IFN-γ-dependent cell-mediated immunity plays a role in protection against acute infection in the mouse model (187). LPS and TNF-α were found to potentiate the effects of IFN-γ in the induction of in vitro macrophage trypanocidal activity (53, 121), although in these studies LPS did not appear to be functioning through stimulation of endogenous TNF production (53). Production of NO appeared to be the principal effector mechanism involved in parasite killing, as demonstrated by elimination of trypanocidal effects in the presence of NMMA (50, 121) as well as the ability of a macrophage cell line that lacks an oxidative burst to kill the parasite in vitro (50). In vivo treatment of susceptible C57BL/6 mice with inhibitors of NO production increased parasitemia and mortality (189).

Both IL-10 and TGF-β inhibited the anti-trypanosomal function of IFN-γ-activated macrophages in conjunction with suppression of NO production (50, 170, 171), and, as observed in other systems, the inhibitory effects of these two down-regulatory cytokines were synergistic (50). An in vivo role for IL-10 and TGF-β in potentiation of infection has been suggested by evidence that mouse strains which are highly susceptible to *T. cruzi* infection produce high levels of these cytokines (170, 171).

The African form of trypanosomiasis lives extracellularly in the blood and lymphatics early after infection but invades the central nervous system in the chronic phase and can cause the fatal neurologic disorder known as sleeping sickness. It has been shown that IFN-γ plus LPS-activated murine peritoneal macrophages exert a cytotoxic effect against blood-derived forms of the extracellular protozoan *Trypanosoma musculi*, a natural parasite of the mouse often used as a model for African trypanosomiases, through an L-arginine-dependent mechanism (192). Again, this effect was reversed by addition of anti-TNF-α, indicating a role for endogenous TNF-α in the activation process. Addition of excess iron also reversed the trypanocidal effect, suggesting that it may be caused by iron loss from critical enzymes in the parasite target. Peritoneal or splenic macrophages from *T. musculi*-infected mice exhibited similar effects. Likewise, IFN-γ plus LPS-treated peritoneal macrophages exerted NO-mediated cytostatic effects against *Trypanosoma brucei* brucei, the causative agent of animal trypanosomiasis, as well as against *Trypanosoma brucei gambiense*, one of the agents of human sleeping sickness, which were reversed by excess iron (193).

**Other parasite targets.** Other protozoan parasites, including *Entamoeba histolytica*, which causes diarrhea and liver abscess, and *Naegleria fowleri*, which can invade the central nervous system with lethal consequences, have also been shown to be susceptible to NO-mediated killing in vitro (36, 45, 104). Likewise, IFN-γ was recently reported to activate thioglycolate-induced murine peritoneal macrophages to kill the microsporidian parasite *Encephalitozoon cuniculi* through an arginine-dependent mechanism (37). In these cases, the lack of substantial information about protective immunity in vivo precludes any judgment at this time concerning the role of this mechanism in resistance to parasite infection. In one in vivo study, however, addition of L-arginine to the diet of nude mice chronically infected with another diarrhea-causing intestinal protozoan, *Cryptosporidium parvum*, was found to reduce fecal oocyst shedding, an effect that was inhibited by N-nitroarginine methyl ester, thus implicating NO generation as the causative mechanism (97). The cellular source of NO in this study was not identified and may not involve macrophages.

**The Unresolved Case of Human Macrophages**

All three isoforms of NOS have been found in humans, and expression of iNOS has been shown for numerous cell types (130). Clinically, treatment with IL-2 has been shown to induce NO production, as measured by urinary nitrite and nitrate excretion (200). The ability of human macrophages to produce NO remains controversial, however (160). Several studies documenting the cytotoxic or cytostatic activity of monocyte-derived macrophages against various microbial targets, including parasites, have reported a lack of concurrent production of measurable amounts of NO (73, 125). There are exceptions, however. In a study of the effect of IFN-γ-treated human monocytes on aspergillar blood forms of the human malaria parasite *Plasmodium falciparum*, incubation in the presence of NMMA was found to at least partially reduce their antiparasitic activity (63). Moreover, it has been reported that pretreatment with IFN-γ plus TNF-α activates macrophages for trypanocidal activity through an NO-dependent mechanism (122).

Several hypotheses have been put forward to explain why cytokine-inducible NOS activity has been so difficult to demonstrate in human macrophages, including blockage by endogenous inhibitors and suppression by immunomodulators (126). Part of the problem may stem from the fact that monocye-derived macrophages are almost certainly physiologically or functionally distinct from the tissue resident macrophages derived from rodents. The tissue-derived cells have been exposed to innumerable stimuli in the process of exiting the circulation, including multiple receptor-ligand interactions and exposure to proinflammatory and inflammatory mediators. Such a complex process is impossible to duplicate in vitro, where the usual monocyte-to-macrophase transformation system involves culturing peripheral blood monocytes for at least 1 week in conjunction with exposure to one or more recombinant cytokines. In those cases (and there are now several) in which NO production by LPS- or cytokine-stimulated human cells has been reported, the amount has usually been smaller than that produced by murine cells and prolonged exposure to cytokines was generally required. Interestingly, a new picture, which suggests a need for other types of signals in the induction of NO production by human cells, may be taking shape. Monocytes incubated with a human colorectal carcinoma cell line were found to produce NO (203). More recent studies have shown NO production by human monocytes following cross-linking of the CD69 integral membrane protein (35) or upon infection with human immunodeficiency virus type 1 (18). The
latter study found that human immunodeficiency virus type 1 infection “primes” human monocytes for enhanced NO production upon stimulation with TNF-α or LPS. Thus, it is possible that human peripheral blood monocytes must receive some additional signal(s) before becoming fully capable of producing NO, which murine tissue-stage cells have already received at some prior point in the inflammatory process.

The possibility must also be considered that human macrophages utilize microbialic mechanisms other than NO production. While production of reactive oxygen intermediates by human cells has certainly been demonstrated, the multiple observations that many parasites are unaffected by these mediators in murine systems and that microbialiic activity proceeds in the absence of superoxide or hydrogen peroxide (50, 161) suggest that this mechanism is not critical. Methods for targeting the availability of intracellular iron other than by NO production, for example, through down-regulation of cellular transferrin receptors, have been postulated as a way of suppressing the growth of intracellular pathogens (196). This mechanism did not appear to be involved in human monocyte-derived macrophage activity against either L. donovani or T. gondii, however (124). Thus, the world awaits identification of some new effector pathway of microbialic activity or the further elucidation of some missing element in the in vitro production of NO by human macrophages, or both, to fully determine the contribution of this mechanism in human immunity.

Activity of Other Cell Types against Parasite Targets

Of course, macrophages are not the only cells with which parasites interact. The facts that (i) many different cell types possess the iNOS pathway and (ii) some form of parasite is known to inhabit virtually every organ and compartment of the body suggest that cells traditionally considered to be outside the immune system network might actually play a larger role in protective immunity against parasites than was previously understood.

Endothelial cells. The observation that endothelial cells are responsive to cytokine signals for NO production suggested that these cells might participate in protection against parasites that possess an intravascular stage. Since schistosomes remain intravascular throughout most of their life cycle in the mammalian host, this was an ideal model with which to test the hypothesis. Studies with murine or human endothelial cell lines showed that these cells are able to kill larval schistosomes via an arginine-dependent mechanism. Both newly transformed and older lung-stage parasites were susceptible to endothelial cell activation for larval killing, and NO production in vitro required two cytokine signals, with combinations of IFN-γ, TNF-α, and IL-1 being the most effective. LPS was also able to provide an adequate second signal (134).

It is tempting to speculate that endothelial cells might be especially important in the effector mechanism of protective immunity manifested against lung-stage schistosomes, since during their migration through the lungs, the parasites are in intimate contact with blood vessel walls. It has been shown by measurement of mRNA levels that the necessary cytokine signals for endothelial-cell activation are generated in the lungs of vaccinated mice (201). In addition, hypertrophy and hyperplasia of endothelial cells of the juxtabronchial arteries of mice vaccinated with irradiated cercariae suggest activation in vivo (134). That such endothelial cell changes are not observed in a strain of mice that does not become protected as a result of vaccination provides strong circumstantial evidence for participation of this pathway in resistance (134).

Hepatocytes. Early studies found that mice treated daily with recombinant IFN-γ or TNF developed lower parasitemias as a result of infection with the murine malaria parasite Plasmodium chabaudi (25). Moreover, injection of TNF, lymphokinin, and IL-1 resulted in measurable levels of reactive nitrogen intermediates in plasma in mice, with TNF producing particularly high levels in malaria-infected animals (153), and the human malaria parasite P. falciparum was found to be susceptible in vitro to killing by nitrite, nitrate, and nitrosothiols (154). Together, these initial observations pointed to a protective role for NO in malaria infection. Indeed, supportive evidence continues to accumulate, although the cellular source of NO in this case appears to be hepatocytes rather than macrophages or endothelial cells.

In the malaria parasite life cycle within the mammalian host, the sporozoite stage, which is injected via the bite of an infected mosquito, rapidly makes its way into the bloodstream and thenceforth to the liver. Liver stage forms of Plasmodium berghei (another murine malaria parasite) were shown to be killed in vitro by IFN-γ-treated hepatocytes, and this activity was inhibited by NMMA or arginase (115, 129). TNF-α was not required, suggesting that in this system, as with leishmanias, the parasite can provide a second signal to allow hepatocyte activation (115). In fact, it has been shown that in vitro infection with P. falciparum results in increased NO formation, even in the absence of added IFN-γ (116). One of the best-studied in vivo models of protective immunity against malaria involves immunization of rodent hosts with radiation-attenuated sporozoites. A recent study showed that hepatocytes of such vaccinated mice became refractory to reinfection and that this protective effect was reversed by treating the animals with NMMA, suggesting that resistance in this model could be at least partially explained by activation of the NO pathway in hepatocytes (132). This conclusion is supported by a study of P. berghei infection in rats immunized with irradiated sporozoites, which demonstrated iNOS activity in hepatocytes within 24 h after challenge infection, with 81% of infected hepatocytes expressing the enzyme by 31 h (85). In this study, neither Kupffer cells nor endothelial cells demonstrated NOS induction. Kupffer cells within the liver could still play an accessory role in this mechanism, however, since hepatocytes can be stimulated to produce large amounts of NO by supernatants from IFN-γ-activated Kupffer cells (33). Examination of the development of resistance in mice rendered deficient in certain aspects of immune response either by in vivo treatment with appropriate monoclonal antibodies or by gene knockout has further contributed to our understanding of the protective mechanisms in this system. Resistance to Plasmodium yoelli (a third type of murine malaria) failed to develop in IFN-γ receptor-deficient mice as a result of initial immunization with irradiated sporozoites (188). Likewise, treatment with substrate inhibitors of NOS, neutralization of IFN-γ, or depletion of CD8+ but not CD4+ T cells in vivo ablated protection against P. berghei in immunized mice (162). Both of these studies also reported enhanced expression of mRNA for iNOS in the livers of normal immunized animals following sporozoite challenge but not in animals rendered specifically immunodeficient and concluded that resistance in this model is mediated through IFN-γ production (presumably by CD8+ T cells), which induces hepatocytes to produce NO that is toxic to liver-stage parasites.

As an interesting aside, repeated immunization with irradiated sporozoites was found to induce a different resistance mechanism, which was IFN-γ and iNOS independent and was likely to be largely antibody mediated (188). This observation is reminiscent of similar findings in mice immunized against S. mansoni by exposure to radiation-attenuated cercariae, when it
was shown that a single vaccination induced a Th1-related, NO-dependent resistance mechanism whereas immune responses became increasingly Th2-like upon multiple vaccinations (20). These observations seem to be telling us something about regulation of Th1 with respect to Th2 reactivity which could be useful in developing vaccination protocols intended to induce a cell-mediated as opposed to an antibody-mediated resistance mechanism.

An additional effect of NO in immunity to malaria may be manifested in decreased parasite transmission, since it has been shown that during periods of schizogony, the infectivity of *Plasmodium vinckei* gametocytes for mosquitoes was substantially reduced by a mechanism that was reversible by treatment with Nω-nitro-L-arginine (120); the cellular source of NO in this case was not identified.

Other cell types. The antiparasitic effects of other cell types possessing the iNOS pathway deserves further study. Cells such as fibroblasts, muscle cells, and epithelial cells are intimately involved in the life cycle of a number of different parasites. It is easy to envision that such cells might play an important role in the host response to parasites such as *Trypanosoma cruzi*, *Toxoplasma gondii*, *Trichinella spiralis*, etc.

**IMMUNE EVASION MECHANISMS AGAINST NO**

As discussed above, many parasites manipulate the host’s own immune apparatus to evade destruction by activated effector cells. That is, they provoke the production of ineffective immune responses which actually antagonize and down-regulate potentially protective responses. In the case of Th1 cell-mediated immunity, this may involve induction of the inhibitory cytokines IL-4, IL-10, and/or TGF-β, which either avert the initial development of an effective Th1 response or prevent effector cell activation. There are other evasive methods available to parasites, however, which allow them to escape the effects of an active immune response in progress. In the case of a cell-mediated response, this may involve switching to an alternative mechanism of energy metabolism to avoid dependence on enzymes that are inactivated by NO (Table 1).

**Toxoplasma Stage Conversion**

*T. gondii* parasites exist in two forms in humans and other intermediate hosts, the rapidly dividing form (the tachyzoite) and the relatively inactive form (the bradyzoite) contained within tissue cysts. Tachyzoites are subject to cell-mediated immune attack, as described above, and newly acquired toxoplasmosis is generally mild in immunocompetent individuals (although development of severe disease involving brain and ocular damage can occur in the immunologically immature fetus, if the mother acquires her first infection while pregnant). In contrast, toxoplasma cysts may persist in muscle, brain, or other tissues of immunocompetent individuals for years. Upon suppression of the immune system, as in patients with AIDS or cancer or in preparation for organ transplantation, long-occult Toxoplasma parasites may become reactivated, resulting in the release of parasites from these cysts. The basis of stage conversion between tachyzoites and bradyzoites has for some time been thought to be influenced by host immunity. Depletion of CD4+ cells or of IFN-γ allowed reactivation of chronic *T. gondii* infection in mice (183, 194). Conversely, vaccination of mice with a tachyzoite surface membrane protein resulted in increased development of intracerebral tissue cysts upon challenge infection (81). Recently, it was shown that spontaneous conversion of tachyzoites to bradyzoites (as measured by expression of stage-specific antigens) was quite low in infected bone marrow-derived macrophages but increased if these cells were treated with IFN-γ or LPS. In these studies, the expression of bradyzoite antigens correlated with decreased parasite multiplication and increased NO production by the host cells. Similar inhibition of parasite multiplication, coupled with enhanced expression of bradyzoite antigens, was observed upon treatment with exogenous NO or with inhibitors of mitochondrial respiration (14). These observations suggest that NO may serve as a molecular trigger of stage conversion, either by selecting for parasites that spontaneously convert to preferential use of NO-insensitive anaerobic metabolism or by actively forcing the parasite to adopt this energy-generating mechanism to survive. This conclusion is supported by a report that in mice chronically infected with *T. gondii*, treatment with anti-IFN-γ or anti-TNF-α results in increased brain damage correlating with decreased iNOS expression and increased expression of genes for tachyzoite-specific antigens (49).

**Schistosome Metabolic Transitions**

It has been widely believed that schistosomes rapidly and permanently convert from the aerobic free-living stage to a form primarily dependent on fermentative metabolism after infection of the mammalian host (reviewed in reference 186). Early experiments confirmed that this transition in energy metabolism was accompanied by decreased susceptibility to NO-mediated killing by activated macrophages (74, 165). It was later observed, however, that the parasite underwent another transient period of susceptibility to macrophage killing between 2 and 4 weeks after infection (140). In this study, by 6 weeks after infection, adult worms were again resistant to macrophages. This observation, inexplicable at the time, has recently been clarified by the observation that migrating schistosome larvae pass through stages at which they are more or less dependent on aerobic respiration for energy metabolism. Thus, the pattern of susceptibility to NO toxicity was found to correlate with that of susceptibility to chemical inhibitors of aconitase and mitochondrial respiration (46, 135). The second phase of vulnerability to NO was observed at around 2.5 weeks after infection in parasites recovered from the livers of immunized mice or from the lungs of mice vaccinated with attenuated cercariae (where they are trapped by the host inflammatory response, as described above).

It is well known that parasitic helminths have the capacity to adapt to different environments by the use of different methods of energy generation. It has been speculated that the shift from aerobic to anaerobic metabolism in schistosomes is not related to availability of oxygen but, rather, to the enhanced activity of the glycolytic enzyme hexokinase (186). It remains unclear why later-stage migrating parasites would revert to greater use of the aerobic pathway, since it means risking exposure to immune attack. It seems likely that a more highly oxygenated environment would be provided during transit through the pulmonary vasculature. In addition, extra energy may be necessary for the motility required to squeeze through tiny vessels. On the other hand, this conversion may be a programmed event related to initiation of a new growth phase, since it is seen in the environment of the portal venous system in parasites derived from unimmunized animals (46, 135, 140). Likewise, the influence of the immune response upon this conversion is not currently understood. It may occur in the lungs of vaccinated (as opposed to naive) mice simply because the challenge parasites are trapped there and cannot move on. Alternatively, the inflammatory milieu of the lungs of vaccinated animals, infiltrated with highly metabolically active cells, may...
present an environment considerably lower in glucose levels than is the normal lung.

OTHER EFFECTS OF NO IN PARASITIC DISEASES

In immunology, as in life in general, too much of a good thing can often be bad. This certainly appears to be the case with NO production, for which small amounts are beneficial in cellular communication and self-defense while unmodulated production becomes autotoxic. For example, NO overproduction has been implicated in autoimmune reactions such as DNA immune complex glomerulonephritis, inflammatory arthritis, and diabetes, as well as in asthma (6, 52, 86, 159). Evidence for a role in septic shock indicates that overproduction of NO in response to microbial infection can also be detrimental (52). The possible role of NO in the pathogenesis of parasitic diseases is just beginning to be understood.

Immunosuppression

Infection with African trypanosomes has long been recognized to suppress host T-lymphocyte responses. This has been demonstrated to be due at least in part to the generation of “suppressor macrophages,” which down-regulate the T-cell proliferative response to trypanosome antigens and mitogens. Recent experiments indicate that NO production is involved in this immunosuppression. Thus, addition of NMMA to cells obtained from T. brucei rhodesiense-infected mice reversed NO production and suppressor cell activity in these cultures (158, 180). Likewise, a possible role for NO in malarial immunosuppression was indicated in experiments showing that NMMA restored the proliferative response of spleen cells from P. vinckei-infected mice to the mitogen concanavalin A, as well as antigen-specific secondary proliferative response of spleen cells from P. chabaudi-infected mice (156). Similar observations have been made for murine Toxoplasma gondii infection (19).

Whether this mechanism is detrimental to the host is not yet clear: it may have a more important beneficial aspect, in that NO can induce a feedback suppression of its own production through its inhibitory effects on proliferating T-lymphocytes and thus control the potential for hyperreactivity. It has been found that NO is particular inhibitory for a Th1-type response (185). Spleen cells from iNOS-deficient mice have been found to contain a higher proportion of CD3+ CD4+ cells, to demonstrate higher levels of proliferation, and to produce higher levels of IFN-γ than do spleen cells from wild-type or heterozygote animals in response to infection or inflammation (195). In many cases, this may be because Th1 cells would be, within the same immunologic milieu, metabolically stimulated both for production of the cytokines necessary for NO induction and for rapid proliferation, thereby rendering themselves more susceptible to the subsequent inhibitory effects of NO on the various enzymes required for energy and DNA replication. The above-mentioned effects of NO on antigen presentation may also be involved.

Vascular Function

Depressed endothelium-dependent relaxation has been described in canine Dirofilaria immitis (heartworm) infection and in lymphatic filariasis (78, 79). In D. immitis infection, this appears to be due to a released parasite product and can be duplicated by serum from infected dogs (93). These results suggest that filarial products may alter NO production by endothelial cells and that this may play a role in the alteration of lymphatic anatomy and function observed in filariasis infection. Thus, while there appears to be significant interplay between the parasite and NO pathways, the possible role of NO in filarial pathogenesis has not been elucidated. Indeed, intriguing preliminary findings (147) suggest that NO plays a protective role in inhibiting the development of Brugia malayi parasites in mice, although the mechanism remains to be clarified.

Cancer

Certain parasitic infections have been identified as risk factors for subsequent development of cancer. These include the proposed association of Schistosoma hematobium infection with bladder cancer, as well as of Schistosoma japonicum and S. mansoni, Opisthorchis viverrini, and Clonorchis sinensis with liver cancer (133). Individuals infected with O. viverrini have been found to excrete higher levels of nitrosoprotein in their urine than uninfected subjects do, suggesting that infected persons have elevated endogenous nitrosation potential. In related studies, NO synthase activity was immunohistochemically demonstrated in the inflammatory cells surrounding parasite-containing bile ducts from the liver of O. viverrini-infected hamsters (133). These observations have led to the hypothesis that increased NO synthesis in response to chronic infection with this liver fluke could result in endogenous formation of nitrosamines from ingested precursors, predisposing to the development of cholangiocarcinoma (133).

Cerebral Malaria

Perhaps the most persuasive argument for a pathogenic role of NO in parasitic disease has been made regarding the proposed link between human cerebral malaria and vascular generation of NO related to overproduction of TNF-α (reviewed in reference 26). Cerebral malaria, which may be characterized by delirium, convulsions, stupor, or coma, has conventionally been explained as resulting from blockage of the cerebral blood supply because of parasitized erythrocytes (3). The argument has been made, however, that the general lack of neurologic impairment observed in those recovering from cerebral malaria does not match with the expected results of long-term oxygen deprivation. According to this argument, the lack of neurologic aftereffects is more consistent with the circumstance of reawakening after anesthesia, a situation which may involve the function of NO in neurotransmission (27).

Studies of cerebral malaria in African children have found that patients with severe falciparum malaria, particularly those with cerebral malaria, demonstrate high levels of TNF-α in plasma (57, 90). A Gambian study (90) suggested that while increased TNF production is a normal response to malaria, excessive levels may predispose to a fatal outcome. A further study in Gambian populations found that individuals homozygous for the TNF2 allele, a variant of the TNF-α gene promoter region associated with high constitutive and inducible cytokine production, have a greatly increased risk for death or severe neurologic sequelae due to cerebral malaria (115). In the P. berghei ANKA mouse model of cerebral malaria, treatment with anti-IFN-γ reduced TNF levels in serum and prevented cerebral lesions (56). Likewise, pentoxifylline, a drug which inhibits TNF-α production, also inhibits cerebral malaria in the mouse model (87). The extrapolation of these observations to human cerebral malaria is not straightforward, however, because the abnormalities in the mouse model result from accumulation of leukocytes, rather than parasitized erythrocytes, in the cerebral blood vessels. It has been reported that lysates of P. falciparum-infected erythrocytes stimulate TNF production by human mononuclear cells, an effect which was
inhibited by a monoclonal antibody recognizing a phosphatidylinositol-like epitope (8). In another study, P. falciparum pigment was found to stimulate the production of high levels of TNF-α and IL-1β by monocytes, and this activity was eliminated by treatment of the parasite pigment with protease (143). TNF-α and IL-1 have been found to synergize to produce increasing levels of reactive nitrogen intermediates in plasma in P. vinckei-infected mice (155), and this production was inhibited by NMMA (153). Collectively, these observations have stimulated the hypothesis that in the case of P. falciparum infection, increased TNF-α levels would stimulate NO production by endothelial cells, vascular smooth muscle cells, macrophages, etc., which might exert a feedback inhibition of NOS activity (62) and disrupt local neurotransmission in the brain (26).

Arguing against this hypothesis is a study which found that treatment with the NOS inhibitors NMMA or N^3-nitro-L-arginine did not reduce cerebral malaria in the P. berghei ANKA murine model (88), a finding that must be considered in light of the inherent difficulties in interpreting this model. More convincingly, contradictory evidence is provided by the report that in Sri Lankan populations, peak TNF levels reached during P. vivax infection surpassed those reported in severe complicated P. falciparum infections, but this was not associated with any cerebral involvement or lethality of infection (80).

**CONCLUSIONS**

The beneficial qualities of cell-mediated immune effector mechanisms have now been demonstrated in many different systems, involving both intracellular and extracellular parasites. These observations should put to rest the convenient but oversimplified paradigm that Th1-type (cell-mediated) immune responses evolved to deal with intracellular microorganisms while Th2-type (antibody-mediated) responses evolved to protect against extracellular pathogens. More important than the cellular location of the target, it appears, is the nature of its dependency on critical metabolic or other pathways inactivated by NO. Thus, both protozoa and helminths are susceptible to killing by NO, and examples in which both types of parasite escape NO attack by converting to the insensitive anaerobic pathway of metabolism have been found. At the same time, it is clear that cellular location of the parasite influences the nature of antigen presentation and T-cell activation. Studies in parasite models have clearly demonstrated that CD8+ T cells can play a previously underappreciated Th-like role in cell-mediated immunity against intracellular pathogens, by producing cytokines such as IFN-γ that direct the function of other cells, including NO-producing effector cells.

With regard to NO production in parasitic infections, it can be concluded that a little goes a long way. The magnitude and time course of NO production appear critical to determining its beneficial versus its detrimental effects. It is clear that overproduction of NO can be destructive not only to its cellular source but also to surrounding tissues. In most cases, however, NO activity seems to be self-limiting because of its short half-life and its feedback inhibitory and immunosuppressive effects. In cases of chronic infection or repeated sensitization, observations in parasite models suggest that the immune system automatically veers toward the Th2 response (which is, of course, associated with its own set of pathologic side-effects). The key to harnessing the protective potential of NO-mediated effector mechanisms will be identification of immunization methods that stimulate potent, localized, short-lived reactions at a time and place where the parasite is fully vulnerable to attack and in the absence of counterregulatory responses.

Results in parasite models firmly illustrate the potential complexity of the immunologic network. Not only are multiple different up-regulatory and down-regulatory cytokines produced by multiple different lymphoid and nonlymphoid cells involved in the effector arm, but also multiple different cell types (including those previously considered to be outside the immune network, such as hepatocytes or endothelial cells) may serve in the effector arm of host defense. This extended schematic is enough to boggle the mind of all but the most enthusiastic graphic artist, and no attempt is made to offer it as an illustration to this chapter. Its complexity must be kept in mind, however, by those molecular immunologists who prefer to reduce events to a subcellular level (which cell?), as well as those of a more applied disposition who may be contemplating the nature of protective immunity with an eye to development of vaccines or immunotherapies. Which responses must be turned on and when? Which responses must be turned off and how? How do different responses interfere or synergize with each other? Such complexity delights the true "biologist," one who appreciates the magnitude of what remains unknown while attempting to make sense of what is currently understood.

Certainly, it is astounding how much has become understood about the function of NO in the last few years. The extent of its role in protection and pathogenesis of parasitic infections will become fully appreciated only as we learn more about its production and regulation in the human immune response.

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