Perspectives Series: Nitric Oxide and Nitric Oxide Synthases

Inducible Nitric Oxide Synthase: What Difference Does It Make?

Carl Nathan
Seaver Laboratory, Division of Hematology-Oncology, Department of Medicine, Cornell University Medical College, New York 10021

Fire sweeps through the brush. In its aftermath, dormant seeds of chaparral, savanna, heath, and scrub begin to germinate in response to a "go" signal in the smoke. Even though smoke-soaked water kills the seeds, in diluted form it triggers their development. The chemical cues are nitrogen oxides (1). This lesson of death and life in the field mirrors comparable events within us, where reactive nitrogen intermediates (RNI)[1] deliver both death- and life-promoting messages. As described in Michel and Feron's introduction to this series (2), RNI include not only nitric oxide (NO), the primary reactive product of nitric oxide synthases (NOSs), but also those species resulting from NO's rapid oxidation, reduction, or adduction in physiologic milieus, such as NO_2, NO_2^-, N_2O_3, N_2O_4, *S*-nitrosothiols, and peroxynitrite ($OONO^-$). In mammals, there is a rough correspondence between toxic and homeostatic functions of NO and its production in large and small quantities, respectively.

The high-output path of NO production is the hallmark of the second isoform of NOS to be cloned, NOS2. NOS2 was named "iNOS" (3) to connote its independence of elevated intracellular Ca^{2+}, the distinguishing biochemical feature primarily responsible for conferring the capacity of this isoform for more sustained catalysis than typically exercised either by nNOS (NOS1) or eNOS (NOS3) (4). Because iNOS is expressed in most cells only after induction by immunologic and inflammatory stimuli, the "i" doubles for "inducible." 5 yr after mouse iNOS cDNA was cloned (3, 5, 6), and 2 yr after the *NOS2* gene was disrupted in mice through homologous recombination (7–9), it is timely to take stock: What does iNOS contribute to mammalian pathophysiology? The complexity of this question has elicited multiple responses addressed to different facets of an answer (e.g., references 10–15). The approach of this *Perspective* is to focus on lessons emerging from iNOS "knock-out" mice. The compound phenotype of these mice (Table I) invites prediction, the limitations of pathophysiologic analysis through gene disruption deserve reflection, and the bottom line demands inspection: In what light does this new knowledge cast iNOS as a potential therapeutic target?

Role of iNOS in control of infection

Fang's masterful review in this space 5 mo ago (15) summarized evidence based on tissue expression and pharmacologic intervention to the effect that mice use RNI to help control a variety of infections. Fang also recounted genetic evidence, based on experiments using iNOS-deficient mice. The latter theme is updated here. Evidence from iNOS-deficient mice paints precisely the picture one would predict for a major pathway of host defense: depending on the infection, the contribution of iNOS to host protection is critical, ancillary, deleterious, or imperceptible. The same can be said of every major weapon in the arsenal of the immune system, reflecting the diversity of infectious agents' metabolic, invasive, and evasive pathways, and the host's need to deploy a variety of weapons in response.

Infections in which iNOS is critical for host survival. By most measures, including proportion of the population infected, duration of infection, and number of resulting deaths, *Mycobacterium tuberculosis* is one of the most successful pathogens of humankind. Nonetheless, the vast majority of infected individuals remain disease-free. Thus, it is of great interest to understand what biochemical mechanisms are used by most immunocompetent individuals to hold the organism in check, or stated differently, to learn what the pathogen must overcome to escape from death or dormancy. Many strains of genetically manipulated mice have increased susceptibility to death from *M. tuberculosis*, including those with disrupted genes affecting CD8 T cell development or encoding T cell receptors, IFN-γ, IFN-γ receptor, or tumor necrosis factor receptor-1. Compared with these, the susceptibility of iNOS-deficient mice appears to be at least as great (16). Since most of the other immune pathways whose role in antituberculous defense has been tested by genetic disruption lead (among other things) to the induction of iNOS, these results suggest that failure to induce iNOS may be sufficient to explain the sensitivity of such mice to infection with *M. tuberculosis.* The most immunodeficient mice previously studied in this setting, those with severe combined immunodeficiency, still display residual resistance as revealed by the further sensitivity manifest upon treatment with glucocorticoids (17). In contrast, iNOS-deficient mice are not rendered any more susceptible when steroid treated; they are already as susceptible as steroid-suppressed wild-type mice (16). Since the tuberculosis-exacerbating effect of corticosteroids is quantitatively indistinguishable from the effect of iNOS deficiency, and corticosteroids suppress iNOS, suppression of

Address correspondence to Carl Nathan, Box 57, Cornell University Medical College, 1300 York Avenue, New York, NY 10021. Phone: 212-746-2985; FAX: 212-746-8536; E-mail: cnathan@mail.med.cornell.edu

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1. *Abbreviations used in this paper:* EAE, experimental allergic encephalomyelitis; iNOS, nitric oxide synthase type 2, whose activity is independent of elevated intracellular Ca^{2+} and whose expression is inducible by infection or inflammation; NK, natural killer; RNI, reactive nitrogen intermediates.

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Table I. Roles of iNOS Inferred from Studies in iNOS-deficient Mice

Setting	Benefit to host	Detriment to host	No major contribution
Infectious disease	↓ *Mycobacterium tuberculosis* ↓ *Leishmania major* ↓ *Listeria monocytogenes* ↓ *Toxoplasma gondii* ↓ *Ectromelia virus*	↑ Influenza virus pneumonitis ↑ Immunosuppression associated with *Mycobacterium avium* infection	↔ *Plasmodium chabaudi* ↔ *Plasmodium yoelii* ↔ *Trypanosoma cruzi* ↔ *Pseudomonas aeruginosa* ↔ *Legionella pneumophila* ↔ *Chlamydia trachomatis*
Inflammation	↓ LPS-induced neutrophil adhesion to endothelium ↓ Leukocyte accumulation in injured colonic mucosa ↑ Closure of excisional wounds ↑ Neovascularization of wounds ↓ Neurologic dysfunction in EAE ↓ Chronic allograft dysfunction ↓ Carrageenin-induced footpad swelling	↑ LPS-induced hypotension ↑ LPS-induced lung damage ↑ Hemorrhage/resuscitation-induced liver damage and activation of proinflammatory transcription factors ↑ Autoimmune vasculitis ↑ Cerebrovascular infarct size ↑ Acute allograft rejection ↑ Allergic airways eosinophilia	↔ LPS-induced liver damage ↔ LPS-induced mortality ↔ Peritoneal leukocyte accumulation ↔ Autoimmune glomerulitis ↔ Autoimmune synovitis

↑, ↓, ↔, increase, decrease, or no major change, respectively, in one or more of the following: microbial titer, host mortality, or tissue response. In some cases, categorizations of responses as beneficial or detrimental to the host are oversimplifications limited to the setting in which the experiment was performed. For example, neutrophil adherence to endothelium can be both beneficial and detrimental. For references, see text.

iNOS may be an important mechanism for the tuberculosis-promoting effects of corticosteroids. In the study cited (16), the host's dependence on iNOS was manifest in all three sites examined—lung, liver, and spleen—as well as in shortened time to death. In similar work from another lab, dependence on iNOS was manifest in liver and spleen but much less so in lung, as assessed by colony counts (18). The reason for the discrepancy is unknown.

Another chronic intracellular pathogen whose control is effected predominantly by macrophages is the protozoan leishmania. The marked susceptibility of iNOS-deficient mice to *Leishmania major* infection was initially believed to be manifest only in the later stages of infection (8). It now appears on closer examination to reflect two distinct roles of iNOS in the wild-type host, which operate at very early and at later time points, respectively (Bogdan, C., personal communication). The more familiar mechanism starts to come into play after several days of infection, when the acquired immune response marshals $CD4^+$ T cells that secrete IFN-γ to activate macrophages. Expression of iNOS by the activated macrophages appears to play an antimicrobial role that is direct as well as indispensable. A fundamentally different role of iNOS was discerned in the first day after inoculation, by which time iNOS has already been induced in an IFN-α/β–dependent manner. Expression of iNOS at day 1 is not sufficiently widespread to kill a substantial proportion of the parasites, but is nonetheless essential for four key elements of the innate immune response: (1) prevention of the dissemination of iNOS-negative cells bearing parasites; (2) responsiveness on the part of natural killer (NK) cells to the NK cell activating factor, IL-12; (3) release by NK cells of IFN-γ; and (4) suppression by IFN-γ of the production of TGF-β, a potent iNOS-suppressing cytokine (Bogdan, C., personal communication). Thus, iNOS appears to play both regulatory and effector roles.

One of the most thoroughly documented antiinfectious roles of iNOS, before the advent of iNOS-deficient mice, was against ectromelia virus, the agent of mousepox (19). Follow-up studies have confirmed that iNOS-deficient mice are substantially more susceptible to the virus in vivo than are wild-type mice (Karupiah, G., personal communication).

Infections in which iNOS plays a beneficial but not a dominant role. The first report of iNOS-deficient mice documented their increased susceptibility to *Listeria monocytogenes* (7). Nonetheless, considerable resistance still remained, as subsequently dramatized by the more profound susceptibility of mice deficient in the transcription factors IRF-2 or ICSBP (20). The latter mice appear to express iNOS normally.

Murine toxoplasmosis provides a fascinating example of a different sort of partially protective function of iNOS, one in which its role depends on the anatomic compartment (21). Proliferation of the protozoan *Toxoplasma gondii* is considerably greater in the brains of iNOS-deficient mice than in wild-type mice, contributing to earlier death. In contrast, in other body compartments, the infection is controlled to the same extent regardless of the presence or absence of iNOS. Although explanted peritoneal exudate macrophages infected in vitro are strictly dependent upon iNOS to kill *T. gondii*, some other mechanism protects the peritoneal cavity from which the macrophages are collected (21).

Infection in which the expression of iNOS is detrimental to the host. Genetic deficiency of iNOS substantially protects mice from death caused by intranasal inoculation with influenza A virus (Karupiah, G., personal communication). This seemingly paradoxical result is fully consonant with an earlier pharmacologic study (22). In this infection in mice, the inflammatory response appears to be a more important cause of mortality than the cytopathic effects of the virus, and iNOS appears to contribute substantially to the inflammation.

During infection with *Mycobacterium avium*, iNOS-deficient mice suffer no greater replication of bacteria in liver and spleen than in control mice, but their splenic lymphocytes are relieved of the inhibition of mitogen responses characteristic

of infected wild-type mice (23). Thus, the predominant action of iNOS in this setting is to cause immunosuppression.

Infections in which no significant effect of iNOS deficiency has been established. Notwithstanding exacerbatory effects reported when infected mice were treated with NOS inhibitors, genetic deficiency of iNOS has had no discernible impact on Chagasic trypanosomiasis (Tanowitz, H., personal communication), or on malaria, as judged by parasitemia after infection with *Plasmodium chabaudi* (Stevenson, M.M., personal communication), or by IFN-γ–mediated protection against liver stage *Plasmodium yoelii* (Tsuji, M., and F. Zavala, personal communication). In several other infections unaffected by iNOS deficiency, there had been little or no preceding pharmacologic basis for expecting the enzyme to be involved. Pathogens in the latter category include *Pseudomonas aeruginosa* (Gros, P., personal communication), *Legionella pneumophila* (Gros, P., personal communication), and *Chlamydia trachomatis* (Perry, L.L., K. Feilzer, and H. Caldwell, personal communication).

Potential relevance of murine studies to human diseases involving infections controlled by macrophages. Because iNOS has been difficult to demonstrate in human macrophages derived in vitro from normal donors' monocytes, the question arises whether there is any clinical significance in the demonstration that tissue macrophages from rodents use RNI to resist certain infections. With hindsight, the controversy seems confined chiefly to the expression of iNOS in healthy donors' mononuclear phagocytes after attempts to activate the cells in vitro. In monocytes or macrophages from patients with a wide range of infectious or inflammatory diseases, iNOS has been more readily detected or induced (for review see reference 14). Human macrophages express iNOS, for example, when collected from the lungs of patients with tuberculosis (24). Inflammatory (but not normal) human alveolar macrophages could be induced in vitro by mycobacterial infection to express iNOS, and they appeared to use iNOS to control the replication of mycobacteria (25). Other clinical settings that have presented with iNOS-positive monocytes or macrophages include alcoholic hepatitis, endemic malaria, rheumatoid arthritis, osteoarthritis, giant cell arteritis, and multiple sclerosis (for review see reference 14), as well hepatitis A under treatment with IFN-α (26). In sum, it is difficult to recreate reproducibly in vitro the macrophage-priming or -activating environments that arise in infected or inflamed human hosts. Given that human macrophages often express iNOS when activated in vivo, the problems encountered in vitro are more appropriately viewed as a deficiency of our culture techniques and immunologic knowledge than as an inadequacy of the cell. Further work is required to determine what contribution iNOS makes in human macrophages when it is fully expressed; most functional studies have addressed the contribution that iNOS does not make, when it is not fully expressed.

Role of iNOS in inflammation

The foregoing findings in infections are mirrored by studies of inflammation induced by nonreplicative stimuli in iNOS-deficient mice. Depending on the setting, the role of iNOS has ranged from enhancing inflammation to retarding it. In fact, the multifaceted nature of some inflammatory syndromes across organs or through time has allowed more than one of these roles of iNOS to be manifest in a single model. As a result, responses to endotoxic bacterial LPS, systemic autoimmunity, and allografts are each discussed under more than one heading below.

Inflammatory settings in which the capacity to express iNOS appears to have a predominantly deleterious effect. The first phenotype associated with iNOS deficiency was resistance to the hypotension induced by injection of LPS in anesthetized mice (7). Similarly, lung damage after LPS injection is markedly reduced in iNOS-deficient mice compared with wild-type mice, as gauged by the lung wet/dry ratio and content of lactate dehydrogenase in bronchoalveolar lavage fluid (Hussain, S.N.A., personal communication). Another form of shock, that caused by hemorrhage and resuscitation, is often followed by severe inflammation in the lungs associated with induction of neutrophil-mobilizing cytokines. In mice and rats, this response is preceded by and probably dependent upon activation of transcription factors NF-κB and Stat-3. Activation of these transcription factors is markedly diminished in iNOS-deficient mice compared with wild-type mice after hemorrhage and resuscitation (Billiar, T.R., personal communication). Thus, in shock states, iNOS is not merely an effector of organ dysfunction, but also a regulator of other effectors. This echoes the role of iNOS in marshaling the innate immune response in leishmaniasis, as described above.

Given the prominence of iNOS in human macrophages infiltrating the intima in giant cell arteritis (27), it is of interest to gauge whether iNOS has the potential to contribute to the development of vasculitis. While there is no reported mouse model of giant cell arteritis, the systemic autoimmune disease that develops in the MRL-*lpr/lpr* mouse includes vasculitis. This vasculitis is markedly ameliorated by iNOS deficiency (28).

Occlusion of the middle cerebral artery induces iNOS in the postischemic brain of wild-type mice beginning after 24 h and peaking at 96 h after occlusion (29). By 96 h, the resulting infarcts are 28% smaller in iNOS-deficient mice than in wild-type mice (29). This is an important extension of the observation that genetic deficiency of nNOS reduces infarct volume measured at 24 and 72 h after occlusion of the middle cerebral artery (30).

In untreated mice, acute rejection of major histocompatibility complex–mismatched, wild-type cardiac allografts is ameliorated in iNOS-deficient recipients compared with wild-type recipients of the same strain (Koeglin, J., and M.E. Russell, personal communication). However, the role of iNOS in acute graft rejection is reversed in chronic rejection, as discussed in the next section.

In a model of allergic airways disease in which immunized mice are challenged with aerosolized ovalbumin, many fewer eosinophils are recovered from the lungs of iNOS-deficient mice (Xiong, Y., and A. Ramsay, personal communication). Levels of IL-4 and -5 are unchanged. It is unclear by what mechanism iNOS promotes eosinophil accumulation in wild-type mice (Xiong, Y., and A. Ramsay, personal communication).

Footpad swelling 24 h after injection of carrageenin is diminished in iNOS-deficient mice compared with wild-type mice (8). It is not known whether the accumulation of fluid, fibrin, or cells is preferentially affected.

Inflammatory settings in which the capacity to express iNOS appears to benefit the host. Administration of LPS to wild-type mice elicits increased sequestration of neutrophils in the lung, their adhesion to endothelium in postcapillary and postsinusoidal venules, and their attachment ex vivo to puri-

fied E-selectin. These responses are markedly exaggerated in mice lacking iNOS (31). Thus, expression of iNOS during sepsis may help retard neutrophil margination, sequestration and activation. Likewise, leukocyte accumulation in the colonic mucosa is more prolonged in iNOS-deficient mice than in wild-type mice after injury by intrarectal instillation of acetic acid (32). Considering that colonic mucosal injury may involve host responses to LPS arising from colonic flora, this finding may be another manifestation of the ability of iNOS to decrease inflammatory neutrophil–endothelial interactions triggered by LPS.

Aseptic wounding induces iNOS. Closure of excisional wounds is delayed by 31% in iNOS-deficient mice compared with wild-type mice (Billiar, T., personal communication). The defect in healing of excisional wounds is quantitatively corrected by a single topical administration of an adenoviral vector containing iNOS cDNA (Billiar, T., personal communication). Likewise, iNOS deficiency markedly interferes with the angiogenesis necessary to sustain survival of a skin flap (Fraulin, F., A. Kane, G. Mitchell, R. Romeo, W. Morrison, and A. Stewart, personal communication). These observations are among the few to demonstrate the requirement for a specific enzyme in wound healing.

SJL mice suitably immunized with myelin basic protein undergo a T lymphocyte–dependent demyelinating syndrome termed "experimental allergic encephalomyelitis" (EAE), widely considered a model of multiple sclerosis. Contrary to expectations based on acute pharmacologic inhibition of NOS in wild-type mice and rats, iNOS-deficient mice backcrossed to the SJL background suffer EAE that is more severe and prolonged (32a). Perhaps this observation reflects the loss of the immunosuppressive action of iNOS at the time of immunization.

In cardiac allografts in immunosuppressed mice, expression of iNOS in parenchymal cells in the grafted heart decreases the severity of chronic rejection, apparently by inhibiting inflammatory cell accumulation and blunting neointimal smooth muscle cell proliferation and the associated graft arteriosclerosis (Koeglin, J., and M.E. Russell, personal communication).

Inflammatory settings in which the host's ability to express iNOS appears inconsequential. Injection of LPS into iNOS-deficient mice primed with heat-killed *Propionobacterium acnes* causes just as much liver damage and margination of neutrophils in the pulmonary vasculature as in wild-type mice (7). Despite the role of iNOS in causing hypotension (7), echocardiographic analysis suggests that the cardiac dilatation in this model is iNOS-independent (33). Most important, iNOS deficiency does not consistently alleviate LPS-induced mortality in conscious mice compared with genetically matched controls (7, 9). In another study, LPS-induced mortality was less in iNOS-deficient mice, but the comparison was made to wild-type mice of a different genetic background (8). Thus, in septic shock, harmful and protective effects of iNOS may contend against each other. Even if the deleterious effects of iNOS predominate, the existence of multiple derangements, each capable of causing death, may obscure the benefit of inhibiting any one such pathway in isolation (7).

Despite the ability of iNOS to interfere with neutrophil–endothelial interaction (31), iNOS deficiency has no impact on the mobilization of leukocytes into the peritoneal cavity after injection of several inflammatory irritants (thioglycollate broth, sodium periodate, and IFN-γ plus LPS) (7).

In the same MRL-*lpr/lpr* mouse model of multisystem autoimmune disease in which iNOS deficiency decreases vasculitis, iNOS deficiency exerts no protective effect against glomerulitis and synovitis (28).

Potential roles of iNOS in homeostasis

The veil of normalcy. As much as iNOS-deficient mice bring home the message that iNOS is sometimes important in shaping the host's response to infection or inflammation, the same mice appear to teach us that the enzyme has nothing to contribute to homeostasis in the unchallenged host. The mice are born to heterozygous parents with the expected Mendelian frequency, indicating the absence of fetal wastage. They gain weight in step with their wild-type littermates, offer no distinguishing features to the pathologist or clinical chemist, and reproduce normally with homozygous-deficient mates (7). This is hardly surprising, since so little iNOS is expressed in the unperturbed host.

Nonetheless, a conclusion that iNOS has no role in homeostasis would be premature, nor should such a conclusion automatically be extrapolated to humans. Within the confines of the vivarium, the mice are spared important physiologic challenges, such as vigorous exercise and changes in climate. Their apparent normalcy may obscure roles played by iNOS in the wild-type host for which alternate mechanisms are called into play when iNOS is congenitally deficient. Finally, more sophisticated studies may reveal subtle phenotypes in iNOS-deficient mice even though they are uninfected and uninflamed. A case in point is described below.

Regulation of transcytosis in pulmonary capillary endothelium. The pulmonary capillary endothelium in iNOS-deficient mice displays a markedly greater number of transcytotic vesicles than in wild-type mice. At rest, the rate of albumin transport out of the pulmonary vasculature in iNOS-deficient mice matches the high level induced in wild-type mice by the activation of complement, and is not further responsive to complement activation (Doerschuk, C.M., personal communication). Apparently, expression of iNOS in the normal, unperturbed mouse exerts a tonic suppressive effect on pulmonary capillary transport function. Two questions arise from these observations: First, by what mechanism do products of iNOS regulate endothelial transcytosis? Second, from what source in normal mice do iNOS-derived products reach pulmonary capillaries? That otherwise normal pulmonary alveolar capillaries register the lack of iNOS suggests that iNOS, rather than nNOS or eNOS, provides a major portion of the RNI to which these cells are constitutively exposed. This surprising notion leads to the following speculation.

Possible contribution of iNOS to the S-nitrosylation of hemoglobin. One of the few settings for seemingly constitutive expression of iNOS in humans is in the respiratory epithelium, especially in large airways (34), as well as in occasional alveolar macrophages (35). The appearance of iNOS in these cells probably reflects their response to inhalation of microbes and irritants. Ozone, for example, induces iNOS (36), and explanted airway epithelial cells lose iNOS, but maintain or regain it in response to substances produced in response to IFN-γ and IL-4 (37). Through the inspired air, NO produced by continuously expressed iNOS in the larger airways could reach alveoli, there to dissolve in the lining fluid, where it is likely to be stored as *S*-nitrosothiols.

Several functions can be envisioned for this strategically

disposed reservoir of iNOS-derived RNI. First, this pool may mediate the constitutive, iNOS-dependent regulation of pulmonary endothelial fluid transport discussed above. Second, some measure of microbial stasis may be achieved by bathing the respiratory mucosa in RNI. A third speculation is inspired by the recent demonstration that when hemoglobin passes through the lung, cysteine 93 in the β chain is charged with a nitroso group, whose discharge in arterioles regulates their diameter in response to the need for flow, as sensed by oxygen tension (38). The source of NO in this homeostatic circuit has not been defined. The hypothesis put forth here is that evolution of mammals in microbiologically active environments has led to reliance upon the continuous expression of iNOS at the portal of the largest, thinnest interface between the outside world and the interior. The resulting accumulation of iNOS-derived RNI in the bronchoalveolar fluid is envisioned as being harnessed for homeostatic functions at distant sites through the allosteric intermediacy of circulating hemoglobin. This hypothesis predicts that tissue pO_2-dependent regulation of arteriolar flow may be blunted in genetically iNOS-deficient individuals, or in genetically normal individuals under field conditions after prolonged, profound pharmacologic inhibition of iNOS.

Possible role of iNOS in uterine physiology. Propagation of the species requires that the gravid uterus relax extensively without stretch-induced activation, and yet commence forceful contractions at term. How is this physiology engineered in humans? In the nongravid uterus, iNOS is undetectable (39). During pregnancy, iNOS is expressed in myometrial myocytes; at the onset of labor, iNOS expression declines precipitously (39). These and related findings noted by Bansal et al. (39) suggest that iNOS may play a role in regulation of uterine contractions in human pregnancy. Although uncomplicated pregnancy is a cytokine-rich, allografted, non–steady state, it is not a disease. Thus the contribution of iNOS envisioned by Bansal et al. (39) would represent a role for iNOS in normal physiology. This speculation regarding a facilitatory role of iNOS in gestation does not dismiss that iNOS may be destructive in the same organ when expressed in other cells, times, or amounts. For example, decidual macrophages express iNOS at the implantation sites of resorbing embryos in mice with high rates of fetal wastage. Administration of an NOS inhibitor forestalls fetal loss, suggesting that iNOS may mediate fetal rejection (40).

The therapeutic horizon

Inhibitors of iNOS. Is iNOS a therapeutic target? The following discussion does not answer the question, but considers criteria that bear on it.

Any molecule whose expression is induced by signals associated with inflammation is likely to be detected in a wide variety of disease states. It is not surprising, then, that iNOS has been detected in people at sites involved by the following conditions: Alzheimer's disease, multiple sclerosis, AIDS-associated dementia, viral uveitis, pulmonary tuberculosis, asthma, lung cancer, pulmonary sarcoidosis, bacterial pneumonia, Crohn's disease, ulcerative colitis, rheumatoid arthritis, osteoarthritis, renal allografts, aortic aneurysms, and psoriasis; in blood monocytes from patients with malaria, rheumatoid arthritis, and alcoholic hepatitis; and in neutrophils from infected urine (for review see reference 14). Nonetheless, expression at the time and place of disease meets only the simplest of criteria that iNOS might constitute a therapeutic target.

Another criterion is the plausibility of iNOS's mechanistic involvement. Here, the cytotoxic and proinflammatory potential of iNOS advances the case for its therapeutic inhibition in those of the diseases discussed above that are not thought to be infectious in etiology, such as Alzheimer's disease, hemorrhagic shock/resuscitation, late-phase vasoocclusive stroke, inflammatory bowel disease, and rheumatoid and osteoarthritis, or in those infectious diseases where the inflammatory effect of iNOS appears to outweigh its antimicrobial effect, such as influenza pneumonia. However, the antiinflammatory role of iNOS emphasizes the possibility of adverse consequences attendant on its inhibition.

The next step in evaluating iNOS as a therapeutic target is the demonstration that genetic disruption of *NOS2* ameliorates a disease that has met the first two criteria. Unfortunately, this forthright standard is problematic. The most serious impediment is the dearth of faithful models of human inflammatory diseases in animals whose genes can be experimentally disrupted. In rodents, how closely does the enteritis caused by gavage with dextran sodium sulfate mimic Crohn's disease, or the rectal instillation of acetic acid recreate ulcerative colitis? To what extent is rheumatoid arthritis produced by intraperitoneal injection of streptococcal cell walls or intramuscular injection of collagen in adjuvant?

Another problem with using unconditional gene disruption to evaluate a therapeutic target is that a gene product may play different roles at different stages in pathogenesis. Conventional knock-out of an enzyme produces a life-long deficiency that differs from pharmacologic inhibition after the onset of disease. In multiple sclerosis, for example, expression of iNOS during the development of autoimmunity may help restrain the expansion of autoreactive T cell clones, while its expression during the destruction of brain may accelerate damage. That mice deficient in iNOS from birth get more persistent EAE than wild-type mice (32a) need not conflict with suggestions of benefit from administration of NOS inhibitors given after onset of disease.

The third criterion is to seek proof of principle in humans by characterizing the phenotype of subjects who are genetically deficient in the target. As yet, no primary state of human iNOS deficiency has been reported. A search for primary iNOS deficiency might profitably begin among well nourished, immunocompetent patients with miliary tuberculosis and a positive family history.

Fourth, one needs to anticipate that mechanism-based toxicity should not be prohibitive. Chronic administration of iNOS inhibitors, for example, might be associated with recrudescence of latent tuberculosis (16) or leishmaniasis (41). Thus, in patients treated with NOS inhibitors, PPD status should be ascertained and the patient monitored in the same manner as is customary with corticosteroid therapy, an iNOS-suppressive, tuberculosis-predisposing modality.

Bearing these obstacles and cautions in mind, it is hoped that the opportunity will arise to test iNOS inhibitors with pharmacologically favorable properties in patients with neurodegenerative disorders, cerebrovascular ischemia/reperfusion, hemorrhagic shock/resuscitation, rheumatoid and osteoarthritis, inflammatory bowel diseases, progressive pancreatic β cell dysfunction (42), and fulminant influenza pneumonitis (22).

Use of vectors containing iNOS cDNA. The fact that wounds require iNOS to heal at a normal rate and that wound healing in iNOS-deficient mice can be reconstituted with iNOS cDNA

suggest the possibility of therapeutic benefit for delivering iNOS by "gene therapy" in some settings (Billiar, T., personal communication). Such approaches have also been contemplated to reduce postangioplasty restenosis (43).

Inhibitors of RNI resistance genes. Now that the role of RNI in some infections in mice has been established, and the relevance to humans judged a possibility, another question arises: How do pathogens catabolize RNI or otherwise defend themselves against their toxic actions? Several new RNI resistance genes have been uncovered recently (15, 44), and RNI resistance properties ascribed to previously reported genes (45). These findings suggest that therapeutic manipulation of the high-output system of NO production need not target only iNOS. To the extent that microbial RNI resistance pathways play a critical role in the host–pathogen relationship, their inhibition might sensitize the pathogen to the host's armamentarium, or to therapeutic agents, such as NO donors, designed to mimic this component of the host's attack. Likewise, when mammalian RNI resistance genes are better understood, their inhibition may enhance the efficacy of tumor immunotherapy.

Conclusion

Over the last 2 yr, more than 120 laboratories have set up colonies of iNOS-deficient mice. Only a handful have had time to complete their studies. Nonetheless, it is already clear that expression of iNOS sometimes makes a profound difference to the course of infection or inflammation in mice. There is as yet no sound experimental basis on which to reject the presumption that iNOS may play a similar role in humans. In both infection and inflammation, iNOS appears to act both as a direct effector and as a regulator of other effectors. The impact of iNOS is potentially dichotomous, and the dichotomy is sometimes manifest at different times or sites in the same experimental setting. These complexities do not preclude experimental therapeutic intervention, but demand caution, whether trials be with iNOS inhibitors, iNOS cDNAs, NO, NO donors, or inhibitors of RNI resistance pathways.

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