Age-Related Decline in Murine Macrophage Production of Nitric Oxide

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Since certain functions mediated by nitric oxide (NO) decline with age, the age dependence of NO production by macrophages from BALB/c mice was investigated. Lipopolysaccharide-, peptidoglycan-polysaccharide-, or interferon-γ-stimulated splenic and peritoneal macrophages from young (1 month old), middle-aged (4–5 months old), and old (6–20 months old) BALB/c mice showed a progressive and marked decline in NO production. This age-related decline in inducible NO extended to C57/BL6 and CB6F1 mice. mRNA for inducible NO synthase (iNOS), the enzyme responsible for inducible NO production by macrophages, also declined with age. Importantly, the reduced NO production by macrophages from old mice could be up-regulated by pretreating the mice with either cholera toxin or concanavalin A. These findings indicate that reduced production of NO by murine macrophages correlates directly with advancing age, likely due to deficient signals or signal transduction responsible for iNOS mRNA and protein generation.

Nitric oxide (NO) is a highly reactive free radical that mediates a multitude of functions, including vasodilation, neurotransmission, and certain immunologic and host defense reactions [1]. The synthesis of NO is accomplished by constitutive and inducible isoforms of nitric oxide synthase (NOS), which catalyze the oxidative removal of the terminal guanidino nitrogen of L-arginine to form NO and citrulline [2]. Whereas constitutive NOS in endothelial cells and neurons promotes the continuous production of small amounts of NO for vasodilation and neurotransmission, inducible NOS (iNOS) is produced by stimulated cells, including macrophages, which results in the generation of potentially cytotoxic amounts of NO [3]. In the mouse, NO has been implicated in macrophage antimicrobial cytotoxicity for a variety of microorganisms, including bacterial, parasitic, fungal, and viral pathogens [4–6] and tumor cells [7]. Since the prevalence of such infections and certain tumors increases with age [8], we investigated whether NO production declines with advancing age, whether this decline is associated with altered iNOS gene transcription, and whether a decline in NO could be reversed by immunomodulation.

Materials and Methods

Mice. Female BALB/c mice (1, 4.5, and 7–20 months old), female C57BL/6 mice (2 and 13 months old), and female CB6F1 mice (1.5 and 15 months old) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and maintained under the same environmental conditions for the duration of the experiment. At the time the animals were sacrificed, there was no evidence of infection or neoplasm. To investigate the effect of in vivo immunomodulation on the production of NO by macrophages from old mice, 20-month-old BALB/c mice were administered a single intraperitoneal injection of either cholera toxin (10 μg; List Biological Laboratories, Campbell, CA), concanavalin A (50 μg, ConA; Sigma, St. Louis), or PBS (Mediatech, Herndon, VA) 3 days prior to harvesting the peritoneal macrophages.

Cells. Peritoneal cavity or spleen cells were isolated from 3–5 mice for each age group by standard techniques, pooled, and suspended in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with gentamicin (50 μg/mL; Mediatech), glutamine (2 mM; Mediatech), and 1-arginine (100 μM; Sigma). The cells were added to 96-well plates (Costar, Cambridge, MA), adjusting the concentration to accommodate the percentage of macrophages in the cavity or tissue; that is, ~10% of spleen cells were macrophages. The macrophages (2 × 10^6/200 μL) were allowed to adhere to the wells, after which the nonadherent cells were removed by washing. The macrophages were cultured for 48 h in the presence of a bacterial product, either peptidoglycan-polysaccharide (5 μg/mL, PGPS; gift of R. B. Sartor, University of North Carolina, Chapel Hill) or Escherichia coli lipopolysaccharide (5 μg/mL, LPS; Sigma), interferon-γ (500 U/mL, IFN-γ; Genzyme, Cambridge, MA), or media alone.

NO measurement. NO was measured in macrophage culture supernatants by quantitating nitrite (NO_2^-), a stable metabolite of NO, with Griess reagent, sodium nitrite serving as a standard [9]. For each plate, the photoabsorbance of a row of control wells containing only phenol red media plus Griess reagent was subtracted from the nitrite-containing wells. Supernatants from triplicate cultures were assayed in triplicate (nine determinations) and reported as the mean concentration of NO_2^- ± SE. The significance of differences between paired values was determined by Student’s t test.

Northern blot analysis. Total cellular RNA was extracted from BALB/c peritoneal macrophages using guanidine thiocyanate–
phenol-chloroform. RNA was separated on a 1% agarose gel containing 1.2 M formaldehyde and transferred to nitrocellulose (BA-S; Schleicher & Schuell, Keene, NH). The blot was probed with a radiolabeled 645-bp HindIII/BamHI fragment of the iNOSL3, a derivative of the macrophage NOS clone (gift of C. F. Nathan, Cornell University, Ithaca, NY) [3], and the density of the bands was evaluated by densitometry.

Results

NO production by splenic macrophages from BALB/c mice of various ages. Equivalent numbers of adherent spleen cells from young (1 month old), middle-aged (4.5 months old), and old (8 months old) BALB/c mice were cultured for 48 h, and the supernatants were assayed for NO$_2^-$ As shown in figure 1A, LPS-stimulated splenic macrophages from young mice produced 2-fold more NO$_2^-$ than did macrophages from middle-aged mice (14.4 ± 4.0 vs. 7.8 ± 2.0 nmol/10$^6$ cells; $P < .001$), and the latter produced 2-fold more NO$_2^-$ than did macrophages from old mice (7.8 ± 2.0 vs. 3.6 ± 1.3 nmol/10$^6$ cells; $P < .001$). When stimulated with PGPS, splenic macrophages from the same young mice produced substantially more NO$_2^-$ than did macrophages from middle-aged mice (13.4 ± 2.8 vs. 8.0 ± 1.1 nmol/10$^6$ cells; $P < .001$). Consistent with the age-dependent decrease in NO production triggered by LPS, the PGPS-stimulated cells from the oldest mice produced the lowest level of NO, which, compared with macrophages from middle-aged mice, was 2-fold less (8.0 ± 1.1 vs 4.0 ± 0.8 nmol/10$^6$ cells; $P = .002$). Unstimulated macrophages from mice of each age group released equivalent low levels of NO$_2^-$ (<2.2 nmol/10$^6$ cells).

Production of NO by peritoneal macrophages from C57BL/6 and CB6F1 mice. Having shown that NO production by BALB/c splenic macrophages in response to bacterial products declines with advancing age, we next determined whether this age-dependent production of NO extends to peritoneal macrophages and to other strains of mice. Similar to splenic macrophages from BALB/c mice, peritoneal macrophages from young (<2 months old) C57BL/6 mice showed a 4-fold higher level of LPS-stimulated NO$_2^-$ production than did peritoneal macrophages from old (≥13 months old) mice (9.1 ± 1.6 vs. 2.3 ± 1.0 nmol/10$^6$ cells; $P < .001$) (figure 1B). In addition, peritoneal macrophages from young (2 months old) versus old (15 months old) CB6F1 mice produced 6-fold more inducible NO$_2^-$ (15.7 ± 1.7 vs. 3.2 ± 1.3 nmol/10$^6$ cells; $P < .001$) (figure 1B). The levels of NO$_2^-$ production by stimulated peritoneal macrophages from old mice of both strains was similar to that of unstimulated macrophages.

Northern blot analysis of iNOS in mouse macrophages. Macrophages from BALB/c mice of increasing age were analyzed for NO production and iNOS mRNA expression. As shown in figure 2A (left), the larger amount of inducible NO$_2^-$ produced by peritoneal macrophages from young mice compared with that produced by peritoneal macrophages from old mice (4.1 ± 2.7 vs. 1.2 ± 0.8 nmol/10$^6$ cells; $P = .001$) corresponded to higher levels of expression of iNOS mRNA in peritoneal macrophages from young versus old mice of the same strain (figure 2A, right). Comparison of the densitometric values for iNOS and GAPDH (a constitutively expressed control gene) mRNA showed that LPS-stimulated peritoneal macrophages from young animals expressed ~6-fold more iNOS-specific mRNA than did such cells from old animals. Thus, the reduction in macrophage production of NO occurred in parallel with a reduction in the expression of iNOS mRNA.

Figure 1. A, Decreased NO$_2^-$ production by splenic macrophages from BALB/c mice of advancing age. Cells from mice at indicated ages were incubated with lipopolysaccharide (LPS, 5 µg/mL), peptidoglycan-polysaccharide (PGPS, 5 µg/mL), or media alone for 48 h, and culture supernatants were assayed for NO$_2^-$ Mid = middle aged; mo = month(s). B, Decreased NO$_2^-$ production by peritoneal macrophages from aging C57BL6 and CB6F1 mice. Peritoneal macrophages from young (<2 months old) and old (>13 months old) C57BL6 and CB6F1 mice were incubated in absence or presence of LPS (5 µg/mL) for 48 h, and culture supernatants were assayed for NO$_2^-$ For A and B, values are mean ± SE of triplicate cultures of pooled cells assayed in triplicate (9 determinations) from 1 of 2 similar experiments.
**Figure 2.** A. Detection of NO\(_2\)^− production and inducible NO synthase (iNOS) mRNA expression by peritoneal macrophages from young (1 month old) and old (8 months old) BALB/c mice. Peritoneal macrophages were incubated in presence or absence of peptidoglycan-polysaccharide (PGPS, 5 μg/mL) for 48 h, and culture supernatants were analyzed for NO\(_2\)^− (left); values are mean ± SE of triplicate cultures of pooled cells assayed in triplicate (9 determinations). Peritoneal macrophages incubated in presence or absence of lipopolysaccharide (LPS, 1 μg/mL) for 4 h were analyzed for iNOS mRNA expression by Northern blot hybridization (right); numbers are ratio of iNOS to GAPDH mRNA densitometric values. B. Enhanced NO\(_2\)^− production by macrophages from old mice pretreated with cholera toxin (CT) or concanavalin A (ConA). BALB/c mice were administered 1 dose of CT (10 μg) or ConA (50 μg) intraperitoneally, and 3 days later, peritoneal macrophages were harvested and analyzed for LPS-stimulated (5 μg/mL), PGPS-stimulated (5 μg/mL), and interferon (IFN)-γ-stimulated (500 U/mL) NO\(_2\)^− production. Values are mean ± SE of triplicate cultures of pooled cells assayed in triplicate (9 determinations).

**Immunomodulation of NO production by peritoneal macrophages from old BALB/c mice.** Since stimulated NO\(_2\)^− production by splenic and peritoneal macrophages from old BALB/c, C57BL/6, and CB6F1 mice was significantly reduced compared with that of macrophages from young animals, we investigated whether NO production by macrophages from old mice could be enhanced. As shown in figure 2B, macrophages from old (20 months old) BALB/c mice that had received only PBS intraperitoneally did not produce increased amounts of NO\(_2\)^− in response to either LPS, PGPS, or IFN-γ. However, macrophages from old animals that had received cholera toxin or ConA 3 days prior to harvesting showed a marked (10- to 40-fold) increase in LPS-, PGPS-, or IFN-γ-stimulated NO\(_2\)^− production (P < .001 for each stimulus). Thus, cholera toxin and ConA appeared to prime peritoneal macrophages from old mice for enhanced production of inducible NO.

**Discussion**

This study shows that the stimulated production of NO by murine macrophages is age-dependent. This finding supports a previous observation by Ding et al. [10] that a decline in IFN-γ-stimulated macrophage production of reactive oxygen and nitrogen intermediates occurs with increasing age. The age-dependent NO production occurs not only in response to cytokines but, as we show, in response to bacterial products as well. This diminished host defense mechanism is not restricted to peritoneal cells or specific mouse strains, suggesting that the effect of age on macrophage NO production may be a common feature of mononuclear phagocytes. Furthermore, the decline in macrophage iNOS mRNA expression with advancing age suggests that the mechanism of the reduction in NO is due, at least in part, to reduced iNOS gene expression.

That the marked reduction in the production of NO by macrophages isolated from mice of advanced age could be overcome by priming the animals in vivo with either cholera toxin or ConA suggests that defective signals or signaling pathways may contribute to declining responsiveness for NO production. Alternatively, reduced NO production by macrophages from aged mice in response to LPS or PGPS or both might also be caused, in part, by macrophage anergy due to repeated interactions with bacterial products during the animal’s lifetime [11].
Reactive nitrogen intermediates, together with oxygen radicals, contribute to macrophage cytotoxic activity toward certain bacteria, protozoan parasites, fungi, and viruses [4–6]. Thus, a reduction in NO production by macrophages from older animals, including humans, could alter the balance between host defense mechanisms and pathogen virulence, thereby increasing host susceptibility to the infectious process. Similarly, NO may contribute to macrophage cytotoxic activity toward tumor cells [7], and the decline in NO with advancing age could contribute to increased susceptibility of aged mice [8], as well as older humans, to certain neoplasms.

Although these findings confirm and extend the observation of Ding et al. [10], Chorinchath et al. [12] recently reported that senescent (24 month old) mice have increased sensitivity to endotoxemia due to tumor necrosis factor-α—mediated NO production. Whereas we studied isolated peritoneal and splenic macrophages, the latter group studied serum levels of NO, using a model of endotoxemia in which multiple cell types, including endothelial cells, and mediators, such as other cytokines, could participate in NO production.

The ability of cholera toxin and ConA to enhance the low levels of NO production by macrophages from old mice suggests a possible role for immunomodulation of macrophage production of this important metabolite. Possible mechanisms of this immunomodulation are currently under investigation. One such mechanism is direct macrophage stimulation by cholera toxin, an agent known to stimulate macrophage secretion of other products, including cytokines [13]. In contrast, ConA could stimulate NO production indirectly, first inducing production of intermediary products, such as tumor necrosis factor-α or interleukin-1, which in turn could induce NO production. However, like many biologic response modifiers, NO has multiple functions, and its up-regulation could potentiate certain inflammatory [14] and autoimmune diseases [15]. Nevertheless, modulation of macrophage NO production may offer a novel approach to therapeutic intervention in infectious and neoplastic processes in the elderly.

References