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Macrophages Produce Nitric Oxide at Allograft Sites

Jan M. Langrehr, M.D., David A. White, B.S., Rosemary A. Hoffman, B.S., and Richard L. Simmons, M.D.

From the Department of Surgery, Free University Berlin, Germany, and the Department of Surgery, University of Pittsburgh, Pennsylvania

Objective

The current study was designed to determine which cytokines produced during an alloimmune response stimulate macrophage nitric oxide (•N = O) production at allograft sites.

Summary Background Data

Previous work has demonstrated that rat sponge matrix allograft infiltrating cells produce more •N = O on stimulation with alloantigen than syngeneic graft-infiltrating cells. Addition of N^G-monomethyl-L-arginine (NMA), an inhibitor of •N = O synthesis, promotes allospecific cytolytic T-lymphocyte effector function.

Methods

Polyurethane sponges were implanted subcutaneously in recipient Lewis rats and injected with 10×10^6 ACI splenocytes. On various days after grafting, graft-infiltrating cells were harvested for *in vitro* study. Adherent macrophages from the graft infiltrating cell population were obtained by a 2- to 3-hour incubation to plastic dishes with subsequent washing to remove nonadherent cells.

Results

Stimulation of unseparated graft-infiltrating cell populations with lipopolysaccharide or interferon-τ resulted in enhanced •N = 0 synthesis by allograft infiltrating cells compared with syngeneic graft-infiltrating cells, early after grafting. Macrophages recovered from an allograft site spontaneously produce more •N = O than macrophages recovered from syngeneic grafts ($p < 0.001$). Significantly enhanced levels of •N = O were produced by allograft macrophages compared with syngeneic graft macrophages on stimulation with lipopolysaccharide or interferon-τ ($p \leq 0.025$).

Conclusions

Nitric oxide appears to be produced in response to the local cytokines secreted by an ongoing rejection reaction. Nitric oxide serves under these circumstances to modulate the alloimmune response.

Nitric oxide (•N = O), produced by macrophages *in vitro,* inhibits lymphocyte proliferation and cytotoxic T-lymphocyte (CTL) generation in response to alloantigenic stimulation in the rat splenocyte culture system. Addition of N^G-monomethyl-L-arginine (NMA), a competitive inhibitor of •N = O synthesis, reduced the NO_2^-/NO_3^- levels (stable end products of •N = O synthesis) in the culture supernatants, resulting in promotion of lym-

phocyte proliferation and cytolytic T-lymphocyte generation.[1] Subsequently, others have confirmed the negative regulatory effect of •N = O on lymphocyte function and showed that the macrophages are the source of •N = O produced in these cultures.[2-5]

To further delineate the significance of •N = O synthesis in the alloimmune response, we used the sponge matrix allograft model and demonstrated that •N = O is produced during an *in vivo* allograft response.[6] Furthermore, restimulation of allograft but not syngeneic graft-infiltrating cells with the sensitizing alloantigen yielded a marked increase in •N = O production, inhibiting an expected second-set proliferation and CTL activity.[7] Efforts to assess •N = 0 synthesis during rejection of vascularized organ allografts showed that serum $NO_2^- + NO_3^-$ levels are elevated during rejection of heart, small bowel, and liver grafts. Administration of the immunosuppressants, CsA or FK506, delayed the elevation in serum $NO_2^- + NO_3^-$ levels until immunosuppression was discontinued.[8] In addition, iron-nitrosyl electron paramagnetic resonance (EPR) signals, characteristic of the reaction of •N = O with iron-containing protein, have been demonstrated in the blood and in the grafted tissue during rat heart and small bowel allograft rejection.[9,10] These results suggest that •N = O is generated at the graft site during rejection. Whether •N = O produced during allograft rejection functions to destroy grafted tissue through its known effect on inhibition of iron-containing protein function[11-13] or whether •N = O is a suppressor molecule, functioning to modulate the immune response, has not been resolved.

Our hypothesis that the cytokines produced by lymphocytes during graft rejection stimulate macrophage •N = O production prompted us to investigate which cytokines are able to induce •N = O production by the graft-infiltrating population. Additionally, •N = O synthesis in response to lipopolysaccharide (LPS) and interferon-τ (IFN-τ) by the adherent macrophages obtained from both syngeneic and allogeneic graft-infiltrating cell populations was compared.

MATERIALS AND METHODS

Animals

Male Lewis ($RT1^l$) and ACI ($RT1^a$) rats, weighing 200 to 225 g, were purchased from Harlan Sprague Dawley, Indianapolis, Indiana. The animals were housed in a specific pathogen-free animal facility and provided with standard rodent chow and tap water *ad libitum*.

Reagents

Reagents were obtained from the indicated companies: minimum essential medium (MEM) (Flow Labs, McLean, VA); Dulbecco's MEM (DMEM) (containing 0.45 mmol/L of L-arginine), fetal calf serum (FCS)(GIBCO BRL, Grand Island, NY); human-interleukin-1 (IL-1β) (Cistron Biotechnology, Pine Brook, NJ); human interleukin-6 (IL-6), murine interleukin-2 (IL-2) and murine-tumor necrosis factor-alpha (TNFα) (Genzyme Inc., Cambridge, MA); rat-interferon-τ (IFN-τ) (Amgen Biologicals, Thousand Oaks, CA); and LPS (*Escherichia coli* 011:B4) (Sigma Chemical Co., St. Louis, MO). N^G-monomethyl-L-arginine was prepared by a modification of the method of Corbin and Reporter.[14] Purity was assessed at > 99% by high-pressure liquid chromatography.

Sponge Allograft Model

The sponge matrix allograft model in the rat was used as previously described.[6,7] Briefly, six polyurethane sponges (2.0 × 0.5 × 0.5 cm) were implanted subcutaneously through incisions in recipient Lewis rats. Immediately after implantation, 10×10^6 allogeneic (ACI) or syngeneic (Lewis) splenocytes were injected into the sponge in a 0.25 mL volume of MEM. On various days after grafting, the sponges were removed and cells that had infiltrated the sponges were obtained by compressing the sponges in three changes of MEM. The cell preparation was treated with TRIS-NH_4Cl to lyse red cells and washed three times.

Peritoneal Cells

Unelicited peritoneal cells were harvested from rats by peritoneal lavage using MEM without serum. Cells were washed and counted.

Culture Conditions (Unseparated Cells)

The medium used for the cultures was DMEM supplemented with 1 mmol/L of sodium pyruvate, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 10 mmol/L of Hepes, 13.6 μmol/L of folic acid, 0.3 mmol/L of L-asparagine, 2 mmol/L of L-glutamine, and 5×10^{-5}mol/L of 2 mercaptoethanol (MLC-DMEM). For experiments designed to assay supernatant NO_2^- by unseparated graft infiltrating cell populations, cells were plated in MLC-DMEM (supplemented with 5% FBS) in microtiter wells at a concentration of 2.5×10^5/well. Cytokines

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Address reprint requests to Rosemary A. Hoffman, B.S., Department of Surgery, Room W1545 Biomedical Science Tower, University of Pittsburgh, Pittsburgh, PA 15261.
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or LPS were added to the wells, achieving a final volume of 0.2 mL. After 48 hours' incubation, culture supernatants were assayed for NO_2^- content. In experiments in which supernatant cytokine levels were assayed, 2×10^6 unseparated graft-infiltrating cells were cultured with 5×10^6 irradiated ACI or Lewis lymph node lymphocytes in 16-mm wells in a final volume of 1.0 mL 5% MLC DMEM. After 24 hours of culture, supernatants were assayed for NO_2^- and various cytokines.

Culture Conditions (Adherent Cells)

Peritoneal cells were plated at a concentration of 1.5×10^6/16-mm well in a 0.5-mL volume and syngeneic and allogeneic graft infiltrating cells were plated at a concentration of 2×10^6/16-mm well in a 0.5-mL volume of 5% FCS-MLC-DMEM. After a 2-hour incubation, the nonadherent cells were removed by vigorous washing and various stimuli were added, achieving a final culture volume of 1.0 mL. On day 2 of culture, supernatants were analyzed for NO_2^- content. Results are expressed as nmol NO_2^- per μg protein.

Total Protein Determination

Adherent monolayers were rinsed with saline, the cells dissolved with 1% sodium dodecyl sulfate and total protein determined using the micro-Lowry assay with BSA as the standard.[15]

NO_2^- Determination

The NO_2^- concentration of culture supernatants was determined by the Griess reaction,[16] using a microplate assay with $NaNO_2^-$ as the standard.

Analysis of Culture Supernatants for Cytokines

Supernatants were assayed for IL-2 content by culturing serial dilution of the supernatant in the presence of the IL-2–dependent cell line, CTLL-2, with rmIL-2 as the standard. IL-6 was assayed similarly using the IL-6-dependent cell line, B-9, and rhIL-6 as the standard. The U/mL concentration for both the IL-2 and IL-6 bioassays was determined by extrapolation of CPM ^{3}H-TdR uptake in the sample to the CPM obtained with the cytokine standard. Interferon-τ was assayed using a rat IFN-τ enzyme-linked immunosorbent assay (ELISA) kit obtained from GIBCO BRL.

Statistical Analysis

The paired Student's t test was used to compare nmol NO_2^-/μg protein in syngeneic *versus* allogeneic graft macrophage cultures.

RESULTS

Evaluation of Alloantigen-Stimulated Graft-Infiltrating Cell Culture Supernatants for Cytokines

Previous results have demonstrated that culture of allograft-infiltrating cells with the sensitizing alloantigen results in induction of •N = O synthesis.[7] To determine which cytokines are produced in these alloantigen-stimulated cultures, syngeneic and allogeneic graft-infiltrating cells were cultured with syngeneic and allogeneic antigen, and culture supernatants were analyzed for NO_2^-, IL-2, IFN-τ, and IL-6. Results depicted in Table 1 indicate that enhanced levels of NO_2^- were detected only in cultures of allogeneic graft cells plus alloantigen. Cultures of syngeneic graft-infiltrating cells plus alloantigen, but not media control cultures or cultures with syngeneic antigen, contained low levels of IL-2. In contrast, supernatants from cultures of allograft-infiltrating cells plus alloantigen contained greatly enhanced levels of IL-2. When culture supernatants were analyzed for IFN-τ, only cultures of allograft infiltrating cells plus alloantigen contained detectable levels of IFN-τ. Assay of culture supernatants for IL-6 showed that both syngeneic and allogeneic graft-infiltrating cells synthesized IL-6 spontaneously (media control), and enhanced levels of IL-6 were detected in cultures of allograft-infiltrating cells plus alloantigen. Thus, the enhanced level of NO_2^- detected in cultures of allograft-infiltrating cells plus alloantigen was associated with enhanced levels of cytokines in the culture supernatants.

Effect of LPS on •N = O Production by Graft Infiltrating Cells

Because LPS is a potent stimulus or co-stimulus of •N = O synthesis by many cell types, including macrophages,[11] hepatocytes,[17] and endothelial cells,[18] the effect of LPS on graft-infiltrating cell •N = O synthesis was examined. Syngeneic and allogeneic graft-infiltrating cells were harvested on various days after grafting and cultured for 2 days in the presence of various concentrations of LPS (Fig. 1). Stimulation of syngeneic and allogeneic graft-infiltrating cells with various concentrations of LPS resulted in enhanced •N = O production by these cells harvested on day 2 after grafting, with maximal •N = O production observed by cells harvested on days 4, 6, and 8 after grafting. The LPS-stimulated •N = O produc-

Table 1. CYTOKINES PRESENT IN SUPERNATANTS OF DAY 6 SYNGENEIC AND ALLOGENEIC GRAFT INFILTRATING CELLS CULTURED WITH ALLOANTIGEN*

	nmol NO_2^-/2.5 × 10^5 Cells	IL-2 (U/mL)	IFNτ (U/mL)	IL-6 U/mL ($\times 10^{-3}$)
Syngeneic graft cells plus				
Media control	3.7 ± 0.2	ND	ND	5.5 ± 0.4
Lewis antigen	4.2 ± 0.1	ND	ND	5.1 ± 0.5
ACI antigen	4.2 ± 0.3	3.9 ± 0.2	ND	4.5 ± 0.3
Allogeneic graft cells plus				
Media control	4.8 ± 0.4	ND	ND	5.8 ± 0.6
Lewis antigen	5.2 ± 0.3	ND	ND	7.9 ± 0.5
ACI antigen	11.5 ± 0.4	26.8 ± 2.0	11.4 ± 0.5	12.6 ± 0.9

* Graft infiltrating cells from Lewis sponges that had been injected with 10 × 10^6 Lewis or ACI splenocytes on day 0 were harvested on day 6. Two × 10^6 graft infiltrating cells were cultured with 5 × 10^6 irradiated Lewis or ACI lymph node lymphocytes in a 1 mL volume. After 24 hours, supernatants were harvested and assayed for NO_2^-, IL-2, IFNτ, and IL-6. Results are expressed as X ± SD of duplicate cultures.

tion by syngeneic cells was concentration-dependent, whereas maximal stimulation of the allogeneic cells obtained on days 4 and 6 after grafting was seen with the lowest concentration of LPS tested (1 ng/mL). Although direct comparisons of nmol NO_2^- present in culture supernatants of syngeneic and allogeneic graft-infiltrating cells cannot be made because of the enhanced spontaneous •N = O synthesis of allograft-infiltrating cells on days 4 and 6 after grafting, some general observations can be made. Higher levels of •N = O were synthesized by day 4 allograft-infiltrating cells in response to lower concentrations of LPS compared with •N = O synthesized by syngeneic graft-infiltrating cells. Because enhanced spontaneous •N = O synthesis was observed by allograft-infiltrating cells on day 4 after grafting, presumably because of exposure to cytokines *in vivo*, it is logical to assume that addition of other •N = O-inducing agents such as LPS would potentiate this reaction. A concentration of 1 μg/mL of LPS also was assayed, but did not induce greater levels of •N = O synthesis by either syngeneic or allogeneic graft-infiltrating cells (data not shown).

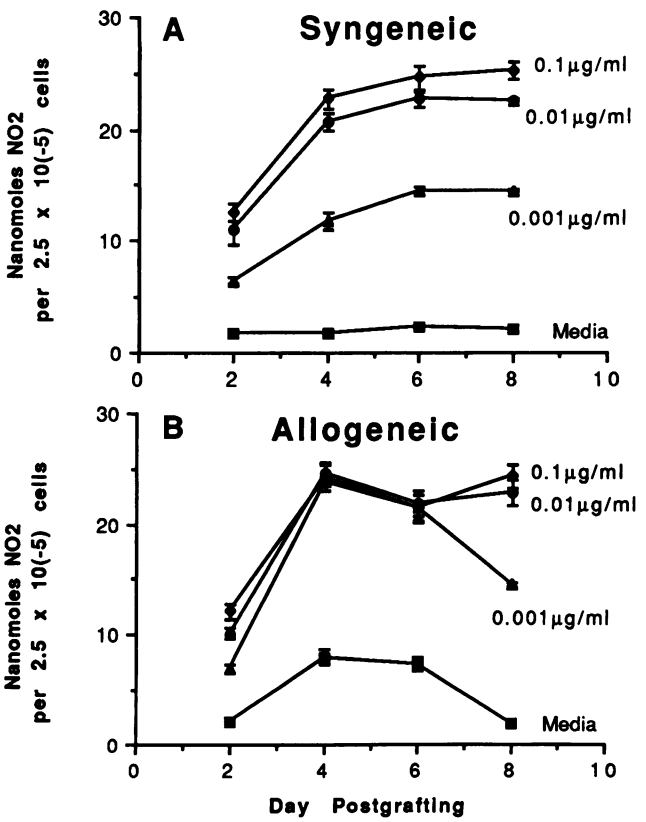

Figure 1. Nitric oxide production by syngeneic and allogeneic graft-infiltrating cells in response to LPS. (A) Syngeneic and (B) allogeneic graft-infiltrating cells were harvested on various days after grafting, and 2.5 × 10^5 cells were cultured in the presence of various concentrations of LPS. After 48 hours of culture, supernatant NO_2^- levels were determined and are expressed as nanomoles per 2.5 × 10^5 cells/0.2 mL volume (mean ± standard deviation of triplicate cultures).

Effect of IFN-τ on •N = O Production by Graft-Infiltrating Cells

Interferon-τ, a T-cell product, is also a potent stimulus of macrophage •N = O synthesis and is produced during alloimmune interactions. Figure 2 depicts the •N = O synthesis by syngeneic and allogeneic graft-infiltrating cells harvested on days 2, 4, 6, and 8 after grafting in response to various concentrations of IFN-τ. Syngeneic graft-infiltrating cell •N = O synthesis induced by IFN-τ was evident on day 2 after grafting and increased on days 4, 6, and 8 after grafting. In contrast, maximal •N = O production in response to IFN-τ was detected by allogeneic cells harvested on day 4 after grafting, and similar levels of •N = O were produced by cells harvested on days 6 and 8 after grafting. Thus, a similar pattern of

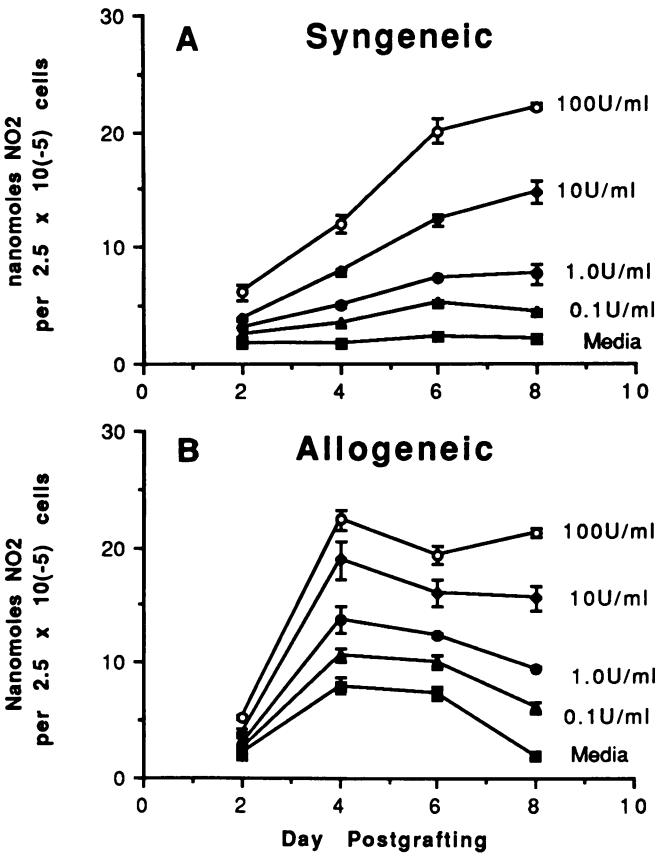

Figure 2. Nitric oxide production by syngeneic and allogeneic graft infiltrating cells in response to IFN-τ. (A) Syngeneic and (B) allogeneic graft-infiltrating cells were harvested on various days after grafting and 2.5×10^5 cells were cultured in the presence of various concentrations of IFN-τ. After 48 hours of culture, supernatant NO_2- levels were determined and are expressed as nanomoles per 2.5×10^5 cells/0.2 mL volume (mean ± standard deviation of triplicate cultures).

induction of •N = O synthesis was seen with IFN-τ as with LPS (*i.e.*, enhanced •N = O production by day 4 allograft cells compared with syngeneic graft cells in response to lower concentrations of stimulus).

Effect of IL-1β, IL-6, and TNFα on •N = O Production by Day 6 Allograft-Infiltrating Cells

To assess the ability of other cytokines to stimulate •N = O production, allograft-infiltrating cells, harvested on day 6 after grafting, were cultured in the presence of various concentrations of IL-1β, IL-6, and TNFα for 2 days (Table 2). Addition of various concentrations of IL-1β and TNFα resulted in a moderate increase in •N = O production, at very high concentrations of cytokines, whereas addition of IL-6 did not induce •N = O synthesis. Interleukin-1β and TNFα added individually have not been reported to induce macrophage •N = O synthesis[19] and the mechanisms of induction in this system is presumed to be by initiation of production of other cytokines that can induce •N = O synthesis.

Comparison of •N = O Synthesis by Adherent Syngeneic and Allogeneic Graft-Infiltrating Cells

The cell population recovered from syngeneic and allogeneic sponge matrix grafts consists of macrophages, lymphocytes, and granulocytes.[7] Addition of cytokines to these cell populations would elicit complex cellular interactions that ultimately might affect the net •N = O synthesis observed. Therefore, the •N = O synthesis by the adherent macrophage population from both syngeneic and allogeneic grafts was compared. To directly compare the amount of •N = O synthesized by these populations, the nmol NO_2^- present in culture supernatants per microgram of adherent protein was determined. Data depicted in Table 3 indicate that supernatant NO_2^- levels in cultures of allograft macrophages cultured in media alone (no *in vitro* stimulation) were greater than NO_2^- levels in cultures of syngeneic graft macrophages cultured in media alone ($p < 0.001$). In addition, at all concentrations of LPS and IFN-τ tested, culture supernatants of allogeneic graft macrophages contained more NO_2^- than syngeneic graft macrophage cultures ($p \leq 0.025$). These results demonstrate that macrophages recovered from an allograft site are primed for •N = O synthesis and produce more •N = O than syngeneic graft macrophages, both spontaneously and in response to stimuli.

Table 2. EFFECT OF IL-1, IL-6, AND TNFα ON •N = O PRODUCTION BY DAY 6 ALLOGRAFT INFILTRATING CELLS

IL-1 (U/mL)	
1	9.2 ± 0.4*
10	9.7 ± 0.4
100	13.3 ± 0.3
IL-6 (U/mL)	
50	9.0 ± 0.6
500	8.5 ± 0.2
5000	8.3 ± 0.3
TNFα (U/mL)	
50	10.9 ± 0.5
500	13.1 ± 0.4
5000	15.6 ± 1.0

* nmol NO_2^- per 2.5×10^5 cells.

Graft infiltrating cells from Lewis sponges that had been injected with 10×10^6 ACI splenocytes on day 0 were harvested on day 6 postgrafting and cultured for 2 days with various concentrations of cytokines as indicated. The culture supernatants were assayed for NO_2^- and the results are expressed as mean ± SD of triplicate cultures. The nmol $NO_2^-/2.5 \times 10^5$ cells cultured with media alone was 9.1 ± 0.1 and in the presence of 1.0 μg/mL LPS was 33.3 ± 1.0.

Table 3. NITRIC OXIDE PRODUCTION BY SYNGENEIC AND ALLOGENEIC GRAFT MACROPHAGES IN RESPONSE TO LPS AND IFNτ

	Syngeneic Macrophages	Allogeneic Macrophages	p Value
Media control	1.1 ± 0.2*	2.4 ± 0.3	<0.001
0.001 μg/mL LPS	2.6 ± 1.4	7.4 ± 2.8	0.002
0.01	11.1 ± 4.1	19.0 ± 8.0	0.025
0.1	15.7 ± 3.7	26.6 ± 8.7	0.001
1.0	18.0 ± 3.6	26.6 ± 3.9	0.001
0.5 U/mL IFNτ	1.6 ± 0.1	3.9 ± 0.2	0.007
5.0	3.2 ± 1.0	8.1 ± 2.8	<0.001
50.0	5.0 ± 1.3	11.5 ± 3.4	0.001
500.0	7.1 ± 0.9	14.4 ± 3.5	<0.001

* nmol NO_2^-/μg of protein.

Cells recovered from syngeneic and allogeneic grafts on day 6 postgrafting were cultured for 2 hours and nonadherent cells were removed by washing. Various concentrations of LPS and IFNτ were added. After 2 days of culture, supernatant NO_2^- was assayed and adherent protein quantitated by the micro Lowry assay. Data are pooled results of triplicate wells from three different experiments and are expressed as nmol NO_2^-/μg protein (X ± SD).

•N = O Synthesis by Unelicited Peritoneal Macrophages in Response to LPS and IFN-τ

Macrophages recovered from either a syngeneic or allogeneic graft have been exposed to the various inflammatory stimuli associated with wound healing. Therefore, •N = O synthesis by unelicited peritoneal macrophages in response to LPS and IFN-τ was assessed. Results depicted in Table 4 indicate that peritoneal macrophages synthesized •N = O in response to both LPS and IFN-τ in a concentration-dependent manner. Addition of IL-6 or TNFα did not result in initiation of •N = O synthesis (data not shown). Thus, macrophages recovered from sponge matrix grafts do not synthesize •N = O in response to different agents than unprimed peritoneal macrophages, but •N = O synthesis is enhanced compared with unelicited peritoneal macrophages.

Table 4. NITRIC OXIDE PRODUCTION BY PERITONEAL MACROPHAGES IN RESPONSE TO LPS AND IFNτ

Media control	0.3 ± 0.03*
0.001 μg/mL of LPS	1.9 ± 0.5
0.01	5.5 ± 1.3
0.1	6.7 ± 1.0
1.0	6.6 ± 0.7
0.5 U/mL of IFNτ	0.5 ± 0.1
5.0	1.4 ± 0.2
50.0	2.8 ± 0.2
500.0	5.0 ± 0.5

* nmol NO_2^-/μg of protein.

Unelicited peritoneal cells were harvested from control rats and cultured for 2 hours. Nonadherent cells were removed by washing. Various concentrations of LPS and IFNτ were added. After 2 days of culture, supernatant NO_2^- was assayed and adherent protein quantitated. Data are expressed as X ± SD of triplicate determinations pooled from two separate experiments.

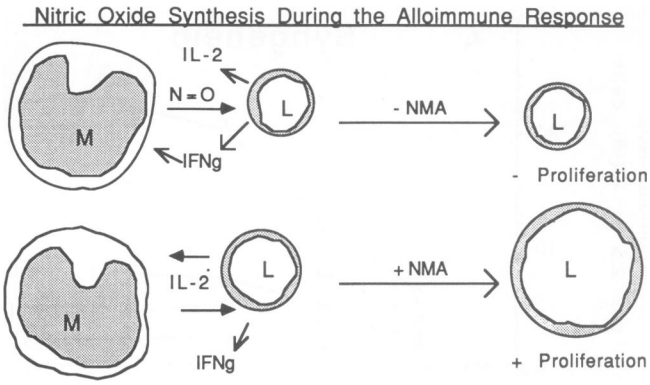

Figure 3. Diagram of proposed mechanism of nitric oxide synthesis during alloimmune response. T-lymphocyte recognition of alloantigen presented by the macrophage results in lymphocyte secretion of IL-2 and IFN-τ. Interferon-τ stimulation of macrophage •N = O synthesis results in inhibition of lymphocyte proliferation. In the presence of N^G-monomethyl-L-arginine (NMA), •N = O synthesis is inhibited and lymphocyte proliferation occurs. L = lymphocyte; M = macrophage.

DISCUSSION

The discovery that activated macrophages synthesize nitric oxide (•N = O) from a terminal guanidine nitrogen atom of L-arginine and thereby induce a metabolic inhibition in cocultured target cells[11–13] has prompted many investigators to further assess this cytotoxic/cytostatic effector molecule. Numerous reports have now documented that cytokine stimulated mouse macrophages exert cytostatic/cytotoxic effects on pathogens such as *Leishmania major,*[20] *Schistosoma mansoni,*[21] and *Toxoplasma gondii*[22] through synthesis of reactive nitrogen intermediates. In addition to the role that macrophages play in host defense mechanisms, such as microbial cytotoxicity, macrophages play a central role in antigen-specific immune responses to foreign proteins and alloantigen. Macrophages process and present antigen in the context of major histocompatibility complex molecules to the responding T-cell, which in turn produces various cytokines. Although some of these cytokines are used by the responding T-cell and result in promotion of lymphocyte effector function, it is now evident that cytokines such as IFN-τ can initiate •N = O synthesis by macrophages, resulting in suppression of lymphocyte effector function. A diagram of the proposed mechanism of induction of macrophage •N = O synthesis during the alloimmune response is depicted in Figure 3. On recognition of alloantigen presented by the macrophage, the T-lymphocyte secretes cytokines, such as IL-2 and IFN-τ.

In the absence of an inhibitor of •N = O synthesis, such as NMA, the IFN-τ–induced •N = O, synthesized by the macrophage, prevents lymphocyte proliferation. In the presence of NMA, when •N = O synthesis is inhibited, T-lymphocytes can use the IL-2 that is produced, and proliferation ensues.

There are species differences in the amount of •N = O produced,[23] as well as the stimuli that induce •N = O synthesis in the rat *versus* the mouse species. When unelicited peritoneal macrophages are used as an indicator system, both LPS and IFN-τ, added singly, stimulate rat macrophage •N = O synthesis, whereas IFN-τ alone is a weak stimulus for mouse peritoneal macrophages and LPS is an ineffective stimulus.[19] Stimulation of mouse peritoneal macrophages with a combination of LPS plus IFN-τ is a potent stimulus for •N = O synthesis. This species difference in •N = O synthesis is also apparent in splenocyte mixed lymphocyte cultures, where, in the rat, addition of NMA is often necessary to detect any proliferative response. In contrast, in the mouse species, addition of NMA enhances the proliferation and allospecific CTL activity observed in the absence of NMA.[1] *In vivo,* using the sponge matrix allograft model, allograft-infiltrating cells in the rat species produce more •N = O, with a more rapid kinetics than in the mouse species.[24] We hypothesize that the enhanced levels of •N = O detected in the rat sponge allograft model is the mechanism whereby less allospecific cytolytic T-cell activity is detected in the rat sponge allograft model than in the mouse model. Thus, we have evidence, both *in vitro* and *in vivo,* that macrophage •N = O synthesis functions to downregulate the immune response to a greater extent in the rat than in the mouse species.

The sponge allograft model has some similarities to models of wound healing used by others. Albina et al.[25] have demonstrated in a wound model in rats that early post-wounding (day 1) when granulocytes are the predominant infiltrating cells, NO_2^- levels in the wound fluid are elevated and that culture supernatants of these granulocytes contain NO_2^-. In contrast, late post-wounding NO_2^- was not detectable in wound fluid, although culture supernatants of cells present in these wounds, predominantly macrophages, contained detectable NO_2^-. The decrease in NO_2^- in late wound fluids may be due to the very low levels of L-arginine, the precursor for •N = O synthesis, in the wound fluid. The low levels of L-arginine may be due to the enhanced levels of arginase activity detected late after wounding.[25] Allograft sponge fluid also contains very low levels of L-arginine, 0.003 mmol/L, compared with serum levels (0.2 to 0.3 mmol/L) (unpublished observation). We have previously shown, however, that culture of allograft-infiltrating cells in L-arginine–free medium results in significantly higher culture supernatant NO_2^- levels than syngeneic graft-infiltrating cells, indicating that sufficient L-arginine must be available *in vivo.*[6] Nevertheless, •N = O synthesis at the wound/graft site may well be regulated by the availability of the substrate, L-arginine.

The experiments examining •N = O synthesis by the unseparated graft infiltrating cells demonstrate that by day 4 after grafting, allogeneic graft-infiltrating cells spontaneously produce more •N = O than syngeneic graft infiltrating cells and that •N = O synthesis can be further enhanced on stimulation with LPS or IFN-τ. Although allospecific cytolytic activity is not detected by day 4 in this model,[7] we speculate that the allosensitization process has been initiated and accounts for the enhanced •N = O synthesis observed. Addition of high concentrations of the cytokines TNFα and IL-1β modestly potentiated •N = O synthesis by allograft-infiltrating cells. Because addition of TNFα and IL-1β to pure macrophage cultures does not result in •N = O synthesis,[19] the mechanism of this enhanced •N = O synthesis may be attributable to complex cellular interactions within the allograft-infiltrating cell population that resulted in enhanced •N = O synthesis. Experiments examining •N = O synthesis by graft macrophages indicate that enhanced levels of •N = O were produced by allograft macrophages compared with syngeneic graft macrophages. Cytokines produced as a result of alloimmune interaction initiated graft macrophage •N = O synthesis, and the macrophages recovered from an allograft site were able to synthesize greater amounts of •N = O in response to LPS and IFN-τ than syngeneic graft macrophages or peritoneal macrophages.

The role of IL-2 in initiation of •N = O synthesis during the alloimmune response remains speculative. Recombinant human IL-2 has not been demonstrated to induce mouse macrophage •N = O synthesis.[19] A recombinant rat IL-2 reagent is not available, and recombinant mouse IL-2 does not elicit rat macrophage •N = O synthesis (unpublished observations). Enhanced levels of IL-2 were present in cultures of allograft-infiltrating cells plus alloantigen compared with syngeneic graft-infiltrating cells. Interleukin-2 is known to potentiate production of IFN-τ by T-cells, and thus an indirect role for IL-2 in alloimmune-induced •N = O synthesis is implied. Other evidence that IL-2 participates in macrophage •N = O synthesis, reported by Jiang et al.,[26] is that macrophage •N = O and TNF synthesis is elicited by IFN-τ, IL-2, and a soluble factor derived from certain tumor cell lines. Thus, IL-2 may have an important costimulatory function under some circumstances.

Although ample evidence exists for the inducible •N = O synthase pathway in rodent macrophages, detection of this pathway in human monocyte/macrophages has not been uniformly successful.[27,28] Nussler et al.[29] have recently demonstrated, however, that human hepatocytes

express the inducible isoform of •N = O synthase. Other reports that document the •N = O synthase pathway in humans are: (1) the demonstration by Hibbs et al.[30] that elevated serum nitrate levels and urinary nitrate excretion were observed in cancer patients receiving IL-2 therapy; (2) the report by Ochoa et al.[31] that serum NO_2^- + NO_3^- levels were elevated in patients receiving IL-2 and CD3-activated T-cell therapy for treatment of end-stage cancer; and (3) the study reported by Ochoa et al.[32] that elevated serum NO_2^- + NO_3^- levels were observed in patients during septic episodes. Thus, the cytokine-inducible •N = O synthase pathway has been documented in humans. The role of •N = O synthesis in various pathophysiologic conditions remains to be elucidated, however.

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