Early Embryo Loss Is Associated with Local Production of Nitric Oxide by Decidual Mononuclear Cells

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Summary

In early embryo loss, the fetus may be considered to be an allograft and, therefore, may be rejected by maternal immunocytes. However, the cytotoxic mechanisms involved are still poorly understood. We have previously shown the involvement of natural killer (NK) cells and mononuclear cells expressing Mac-1 (CD11b) and F4/80 in resorbing compared to nonresorbing embryos. In this study, the role of nitric oxide (NO) in the mechanism of early embryo loss was studied. Pregnant CBA/J females mated with DBA/2 males (20–30% early embryo loss) and CD1 females mated with CD1 males (5–10% early embryo loss) were studied on days 8, 10, and 12 of gestation. Cells from the implantation sites of individual embryos were tested for the production of nitrite and nitrate with or without in vitro challenge with lipopolysaccharide (LPS) to determine whether decidual macrophages were primed in situ. On day 12 of gestation, when resorption was clearly visible, resorbing embryos showed more than a fivefold increase in both basal- and LPS-induced nitrite and nitrate production compared to nonresorbing embryos in both mouse strains tested, indicating that the decidual mononuclear cells were primed. Furthermore, more than 20% of CBA/J embryos showed a significant nitrate release on days 8 and 10 of gestation before any signs of embryo cytopathology. This percentage corresponded to the spontaneous resorption rate seen in CBA/J female × DBA/2 male matings. Similarly, 4% of the embryos from pregnant CD1 mice on days 8 and 12 of gestation produced a significant amount of nitrate, which again correlated with the low incidence of resorption observed in these mice. Using immunohistochemistry, the presence of inducible nitric oxide synthase (iNOS) was detected at implantation sites. Furthermore, decidual cells positive for both iNOS and the macrophage marker Mac-1 were demonstrated in implantation sites by double immunostaining. This strongly suggests that decidual macrophages could be the cellular source of NO production. Aminoguanidine, a selective inhibitor of the iNOS, inhibited the in vitro production of nitric oxide by cells isolated from individual implantation sites, and more strikingly, significantly reduced early embryo losses in CBA/J females mated by DBA/2 males when given orally or parenterally to the gravid females starting on day 6 of gestation. In addition, aminoguanidine-treated pregnant mice showed a significant increase in average litter size when the pregnancies were allowed to proceed to term. The results presented strongly suggested a role for NO as an effector molecule in mediating early embryo loss and showed that the in situ activation of decidual macrophages was an early event preceding spontaneous abortion.

The mechanisms of early embryo loss observed in humans and many other animal species are not yet fully understood. Most of our knowledge regarding the mechanism of early fetal resorption has been obtained by studying the cell biology of resorption prone matings of CBA/J × DBA/2 mice. The natural resorption rate in CBA/J females when mated by DBA/2 males is 20%–25% (1). This could be further increased to 60% of the litter if the females were injected with poly I:C, a synthetic double-stranded RNA molecule capable of inducing IFNs that activate innate resistance (2). The enhancement mechanism of early embryo loss by poly I:C showed a similar time course and cytopathology to that observed in the untreated CBA/J females mated with DBA/2 males (3, 4). By using this experimental model, we have previously shown that NK cells were associated with early fetal rejection. NK cells heavily infiltrated some, but not all, embryos on day 8 of gestation (5). Furthermore, injecting pregnant CBA/J mice with anti–asialo-GM1, a rabbit anti-NK cell antiserum, significantly reduced the incidence of resorption (6). Recently, we reported the association of maternal macrophages with early embryo loss (3). Implantation sites from potentially

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resorbing embryos showed a significant increase in decidual Mac-1– and F4/80–positive cells compared to nonresorbing embryos. In addition, other researchers have shown that normal embryos show significant numbers of leukocytes in the decidual and placental tissues (7–9). Taken together, these results suggested a role for macrophages and NK cells in mediating early fetal rejection. However, these studies did not address the cytolytic effector mechanism involved and the state of activation of the infiltrated leukocytes.

The involvement of cytokines such as TNF-α, IFN-γ, and IL-2 in the mechanism of fetal rejection has been reported (10). IL-2 induces the production of IFN-γ by NK cells (11). IFN-γ secreted by NK cells primes macrophages for TNF-α and nitric oxide (NO)[1] production (12–14). In fact, these cytokines were shown to increase the frequency of resorption in CBA/J female × DBA/2 male matings (10). Direct evidence of embryotoxic effects from these cytokines in mediating embryo loss, however, is still lacking.

In this study, we investigated the role of macrophage activation in early embryo loss as determined by the release of nitric oxide, a macrophage–derived cytotoxic molecule, synthesized by an enzyme called NO synthase (NOS). NO is a short-lived mediator that can be induced in a variety of cell types and produces many physiologic and metabolic changes in target tissues and cells (15). At least three isotypes of the NOS enzyme have been identified, including two constitutive isotypes (cNOS), found in endothelial and neuronal cells, and an inducible isotype (iNOS), present in activated macrophages and neutrophils (16). The iNOS is inducible by IFN-γ, TNF-α, IL-1, and LPS (17). Unlike the constitutive form, the inducible isotype binds calmodulin with high avidity and hence it is essentially independent of added calmodulin for its action (18). Calmodulin copurifies with the iNOS after boiling in SDS, thus calmodulin acts as a constitutive subunit of the iNOS (18). Both isotypes convert L-arginine into L-citrulline and NO, and both are inhibited by many L-arginine analogues such as *N*-monomethyl arginine and aminoguanidine (AG) (19, 20). The latter is very much more inhibitory to the iNOS rather than the cNOS (21–23).

Two signals are needed for an effective synthesis of NO by macrophages: a priming signal that is usually mediated by IFN-γ, provided by activated T cells and/or NK cells, as well as a triggering signal that is delivered by either TNF-α or LPS (17). The rationale behind these studies proposes that decidual infiltration by activated NK cells and macrophages provides the necessary components for the priming of decidual macrophages and release of macrophage–derived cytolytic factors. This study shows that decidual macrophages are primed in vivo during early pregnancy and that activated NO production is associated with early embryo loss.

Materials and Methods

Experimental Animals. DBA/2 males and CD1 mice were purchased from Charles River (St. Constant, Quebec, Canada), and CBA/J females were purchased from The Jackson Laboratory (Bar Harbor, ME). The housing and handling of the experimental animals were in accordance with the guidelines of the Canadian Council for Animal Care. Four female mice were housed with a single male mouse and were checked daily for the presence of a copulatory plug. The day of a mating plug's appearance was arbitrarily designated as day 0. Mice were then killed by cervical dislocation on days 8, 10, and 12 of pregnancy. To increase the basal incidence of resorption, a separate group of CBA/J females were injected intraperitoneally with 30 μg of poly I:C on day 6 of gestation. Treatment with poly I:C has been shown to increase fetal resorption up to 60% (2).

Primed macrophages were obtained from the spleen of nonpregnant mice 5 d after they were injected with 1.4×10^8 viable Bacille Calmette-Guérin (BCG).

Preparation of Decidual Cell Suspension. The uteri were surgically removed and the number of viable implantation sites together with the number of resorbing embryos were counted. For day 10 and 12 embryos, the uterine tissues were peeled off and the implantation sites were separated from the embryos. On day 8, both the embryo and the implantation sites were used as sources of cells because of the difficulties in separating them. Using a sterile Richard utility blade, individual implantation sites were minced in 1 ml of cold HBSS into very small pieces. The cell suspensions together with the tissue debris were filtered through a 93-μm nylon mesh (Thompson B & SH, Montreal, Quebec, Canada). The filtrates were centrifuged at 200 *g* for 10 min in an IEC centrifuge (International Equipment Co., Needham Heights, MA). Contaminating red blood cells were disrupted in sterile lysing buffer (0.15 M NH_4Cl) for 3 min at room temperature. The cells were then washed in HBSS. The last wash was done in medium free of nitrite, nitrate, and phenol red (Sigma Immunochemicals, St. Louis, MO), supplemented with L-glutamate (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% FCS (ICN, Montreal, Quebec, Canada). The pellet was then resuspended at 1×10^6/ml in nitrate-free MEM. Generally, >90% of the cells were viable as shown by trypan blue exclusion, and ~10% stained with Mac-1 and F4/80 antibodies (data not shown). The cell suspension was then aliquoted to a 96-well flat-bottomed culture plate (100 μl per well) (Flow Laboratories, McLean, VA), and the cells were incubated with either 50 μl of medium containing 10 μg of LPS (055:B5 *Escherichia coli*; Sigma) or with medium alone. In some of the experiments, 1 mM of AG (Sigma) was added in a 50-μl vol of medium. After an overnight incubation at 37°C in a 5% CO_2 incubator, the supernatants were harvested and tested for the presence of nitrite and nitrate.

Single and Double Immunohistochemistry. In this experiment, frozen sections (8-μm thick) were prepared from day 8 uteri, air dried, and fixed in 2% paraformaldehyde (Fisher Scientific, Montreal, Quebec). To reduce nonspecific staining, sections were incubated with normal goat serum (Vector Laboratories, Burlingame, CA) for 20 min at room temperature. In single-labeled immunostaining, washed sections were then incubated with rabbit to antibody to murine iNOS (1:1,000) (Transduction Labora-

[1]*Abbreviations used in this paper:* AG, aminoguanidine; BCG, Bacille Calmette-Guérin; cNOS, constitutive nitric oxide synthase; DAB, diaminobenzidine; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase.

tories, Lexington KY) overnight at 4°C. Sections were equilibrated at room temperature for 1 h and then incubated with biotinylated anti–rabbit IgG antibody and later with avidin/biotinylated horseradish peroxidase complex (ABC) used according to the manufacturer's instructions (Vector Laboratories). The colour was developed for 5 min with peroxidase substrate, diaminobenzidine (DAB) (Sigma), and counterstained with Mayer's hematoxylin (Sigma). Sections were then mounted with Crystal/Mount (Biomedia, Foster City, CA).

In double-labeled immunostaining, slides were simultaneously incubated with rat anti–Mac-1 antibody (American Type Culture Collection, Rockville, MD) and rabbit anti-iNOS antibody. Washed sections were incubated with alkaline phosphatase–conjugated goat anti–rat IgG (Sigma) together with horseradish peroxidase–conjugated goat anti–rabbit IgG (Sigma). The sections were first stained with alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma). Levamisole (Sigma) was added to the substrate buffer to reduce the activity of the endogenous placental phosphatase. The washed sections were then incubated with the peroxidase substrate DAB. Sections were then mounted with Crystal/Mount (Biomedia). In both staining methods, endogenous peroxidase was quenched with a 15-min incubation in 3% hydrogen peroxide solution (Sigma) in 100% methanol (American Chemicals, Montreal, Canada) after incubation with the primary antibody. As a negative control, sections were incubated with the second antibodies and substrate without exposure to the primary antibody or with the addition of a primary isotype control antibody.

Preparation of Spleen Cell Suspension. Spleen cells from mice that had been injected 5 d previously with BCG were prepared by mincing the spleen in 5 ml of RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FCS, 2 mM L-glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspensions were sedimented for 10 min on ice. The tissue debris was discarded and the cell suspension was centrifuged for 5 min at 200 *g*. The resulting pellet was then washed three times with HBSS. The cells were resuspended in RPMI at a concentration of 1×10^7/ml and 100-μl aliquots were placed in each well of a 96-well flat-bottomed plate in the presence or absence of 10 μg of LPS at 37°C and 5% CO_2.

Assay for NO. NO produced by activated macrophages accumulates as nitrite (NO_2^-) and nitrate (NO_3^-) in the culture supernatant. Nitrite in 50 μl of the culture supernatants were assayed with Greiss reagent (24). Briefly, equal amounts (50 μl) of the supernatant and Greiss reagent (1% sulphonamide in 2.5% H_3PO_4, and 0.1% [1-naphthyl]ethylenediamine in water) were allowed to react in flat-bottomed 96-well culture plates with gentle mixing for 10 min at room temperature. The colored product was read on a Titertek Multiskan (Flow Laboratories, McLean, VA) reader at 540 nm using 670-nm readings as reference wavelength to compensate for nonspecific absorbance. The concentration of nitrite in the samples were determined as micromoles of NO using the standard curve for nitrite included in each assay. Where total NO production as both nitrite and nitrate were assayed, nitrate was first reduced to nitrite using a kit obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Briefly, the reduction of nitrate to nitrite was achieved by using nitrate reductase enzyme purified from *Aspergillus* in the presence of nicotinamide dinucleotide phosphate (NADPH). NADPH solution (20 μl), prepared by dissolving 0.5 mg of NADPH in 400 μl ddH_20, was added to 50 μl of the supernatant followed by 9 μl of purified nitrate reductase (5 U/ml). The mixture was incubated for 20 min at room temperature and the reduced nitrate was then detected by the Greiss reagent as described above. The concentration of nitrate was deduced from a nitrate standard curve. In effect, this assay measured total NO production.

Treatment of Mice with AG. Pregnant CBA/J females mated by DBA/2 males were injected on day 6 with 30 μg of poly I:C. From days 6–10, the mice were injected twice daily with 6 mg of AG intraperitoneally, while the control mice received saline. Alternatively, to avoid the trauma associated with injecting mice, AG was added to the drinking water (0.6 g/100 ml) on days 6–10. The mice were killed on day 12, and the number of implantation sites together with the number of resorbing embryos were counted as described above. The proportion of resorbing embryos was presented as a percentage, as derived from the formula 100 × resorbing embryos/(viable + resorbing embryos). A separate group of mice were allowed to go to term, and the number of delivered embryos were enumerated and examined for gross morphologic defects.

Results

Production of NO Is a Sensitive Indicator of Macrophage Priming. To demonstrate the utility of NO as an indicator of the priming of macrophage in vivo, splenocytes were isolated from BCG-primed mice and challenged with LPS in vitro. It was apparent that the augmented ability of macrophages to produce NO was a sensitive measure of macrophage activation. Resting macrophages produced little NO whether or not they were challenged with LPS in vitro, while BCG-primed splenic macrophages produced significant amounts of NO when challenged with LPS compared to the BCG-primed macrophages alone (Fig. 1). Furthermore, the quantity of nitrite produced was directly proportional to the number of spleen cells incubated (data not shown). These results showed that both a priming signal and a triggering signal were needed for macrophages to

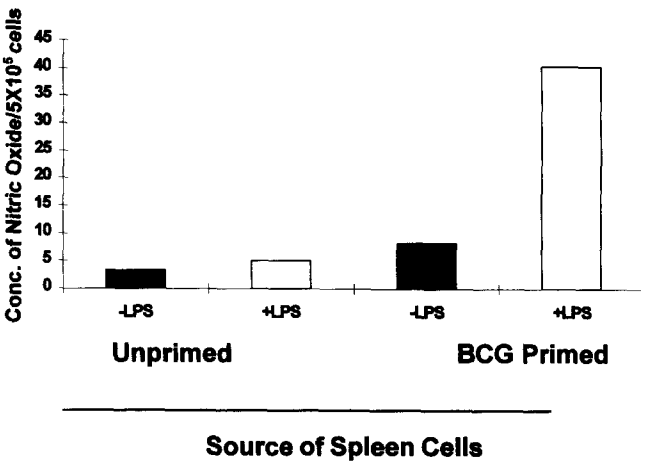

Figure 1. Nitric oxide production by BCG-primed spleen cells. Mice were injected intraperitoneally with 1.4×10^8 BCG organisms or PBS, and 5 d later, spleen cells were collected (1×10^7/ml) and challenged with LPS in vitro. Culture supernatants from 1×10^6 spleen cells per well incubated with 10 μg of LPS per well were harvested and assayed for NO accumulation as nitrite using the Greiss reagent. *Open bars*, nitric oxide production by cells challenged with LPS; *closed bars*, cells challenged with medium alone. Results expressed as μM NO/5×10^5 cells.

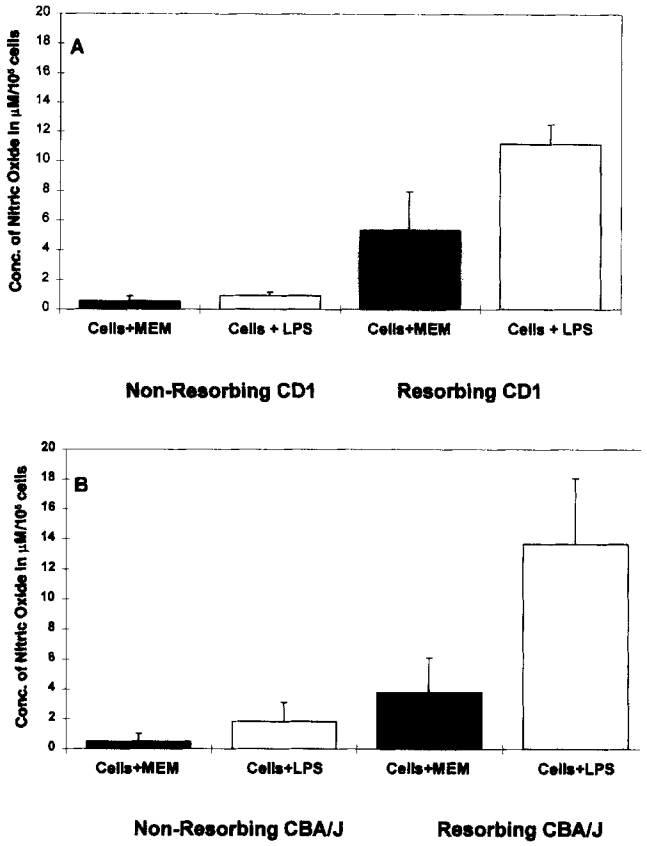

Figure 2. Nitric oxide production by cells from implantation sites taken from CBA/J (*A*) and CD1 (*B*) resorbing and nonresorbing embryos on day 12 of gestation. Pregnant female mice were killed on day 12 of gestation, and cells (10^5 in 100 μl) from individual implantation sites of resorbing and nonresorbing embryos were prepared for in vitro challenge with LPS. Culture supernatants were assayed for the presence of NO as nitrite, and the results were expressed as micromoles of NO in the culture supernatant. *Open bars*, NO produced by LPS-challenged cell cultures; *closed bars*, cells cultured with PBS. Results are presented as means ± SD for assays performed using 4 CBA/J and 11 CD1 mice.

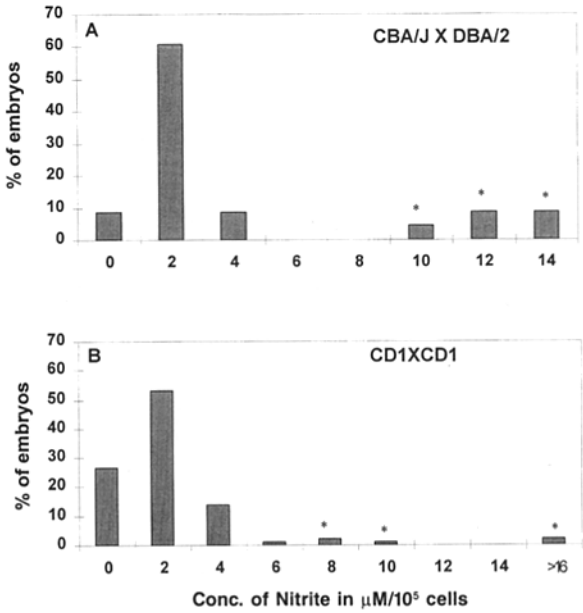

Figure 3. The frequency of distribution of day 12 embryos versus nitrite production. Cells from implantation sites from resorbing and nonresorbing embryos were individually tested for NO production as nitrite on day 12 in 4 CBA/J females mated with DBA/2 males (*A*), and 11 CD1 females mated with CD1 males (*B*). The results are expressed as micromoles NO in the culture supernatant. Bars indicate the number of embryos producing between 0 and 16 μM of nitrite by 10^5 decidual cells by 2-μM intervals. *Groups of day 12 embryos that were all resorbing.

produce NO. Therefore, the production of NO by mononuclear cells in response to in vitro challenge with LPS could be used as a sensitive indicator of the priming of macrophages.

NO Is Produced by Decidual Cells from Resorbing Embryos. In this study, we investigated the role of NO as an effector molecule in the mechanism of early fetal rejection. Assays of NO production by cells at the implantation sites from CBA/J female mice indicated that decidual mononuclear cells were primed in vivo and were responsive to challenge by LPS (Fig. 2). Furthermore, decidual cells from resorbing embryos on day 12 of gestation produced significantly more NO than nonresorbing embryos. Likewise, decidual cells from CD1 mice produced similar concentrations of NO, and moreover, resorbing embryos from CD1 female mice showed elevated quantities of NO (Fig. 2). NO production, therefore, appears to be a good marker for embryo resorption.

The results documented in Fig. 2 showed that implantation sites taken from day 12 resorbing embryos of both CBA/J (Fig. 2 *A*) and CD1 (Fig. 2 *B*) produced significantly more NO as compared to nonresorbing embryos. When the data was plotted as a frequency histogram, the production of nitrite by individual embryos from both CBA/J and CD1 females on day 12 of gestation showed a distinct bimodal distribution (Fig. 3). This bimodal distribution was also seen when day 12 CBA/J embryos were

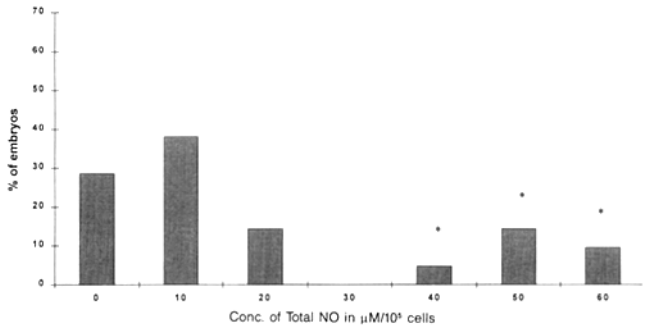

Figure 4. The frequency distribution of day 12 CBA/J embryos vs total nitric oxide production (nitrite plus nitrate). Cells from implantation sites from resorbing and nonresorbing embryos taken from four CBA/J females were individually tested for total NO production as both nitrite and nitrate. The results are expressed as micromoles of NO in the culture supernatant. Bars indicate the number of embryos producing between 0 and 70 μM of NO by 10^5 decidual cells by 10-μM intervals. *Groups of day 12 embryos that were all resorbing.

Table 1. *Production of Nitrate by Embryos from CBA/J × DBA/2 Matings between Days 8–12 of Gestation*

	CBA/J mice tested	Implantation site per mouse	Number of embryos that produced nitrate without LPS*	Number of embryos that produced nitrate with LPS*	Percent of embryos with significant nitrate production with LPS
Day 8	5	6.2 ± 1.3	3	9	29.0%
Day 10	4	8.5 ± 2.0	1	6	17.6%
Day 12	4	6.3 ± 1.3	1	5	20.0%

*Nitrate production above the upper 95% confidence limit (UCL 95%) established for day 12 nonresorbing embryos (>22 μM/10^5 cells) was considered significant.
Mice were killed on day 8, 10, or 12 of gestation. Embryos were minced in nitrite- and nitrate-free medium, and the cell suspensions were challenged with LPS in vitro for 24 h. Reduction of nitrate to nitrite was achieved using nitrate reductase in the presence of NADPH.

tested for the production of nitrate (Fig. 4). It was clear from this data, however, that nitrate was a more sensitive indicator of total NO production than nitrite alone, and nitrate was measured for subsequent assays.

All the resorbing embryos from pregnant CBA/J mice produced between 30 and 70 μM of nitrate per 10^5 decidual mononuclear cells. All the nonresorbing embryos produced between 0 and 30 μM nitrate. The nitrate production by the nonresorbing embryos was therefore used to define the limits of normal nitrate production for the analysis of embryos at earlier stages of development. The day 12 nonresorbing embryos produced an average of 11.23 ± 6.12 μM of nitrate and the upper 95% confidence limit for nitrate production was computed as 22 μM per 10^5 decidual cells. Using this value, nitrate production by individual embryos on days 8 and 10 of gestation were categorized as being normal or significantly increased. Table 1 shows data for CBA/J × DBA/2 matings in which an average of 23% of embryos showed increased nitrate production between days 8 and 12. Similarly, 3% of embryos from CD1 × CD1 matings showed elevated levels of nitrate production between days 8 and 12 (Table 2). Therefore, in both mating combinations, the production of significant concentrations of nitrate were associated with embryo loss. More interestingly, decidual cells from CBA/J × DBA/2 embryos on days 8 and 10 produced significant amounts of nitrate, even in the absence of LPS, suggesting that NO-produced damage may have already begun as early as day 8 in these embryos. Increased NO production by cells at the implantation sites therefore preceded visible embryo resorption.

Expression of iNOS Protein by Decidual Mononuclear Cells. To investigate the presence of iNOS in pregnant mouse uteri, implantation sites from CBA/J females mated with DBA/2 male mice were stained with rabbit anti–mouse iNOS antibody. Fig. 5 *a* shows a representative view of a day 8 embryo, where positive cells were observed in the

Table 2. *Production of Nitrate by Embryos from CD1 × CD1 Matings between Days 8–12 of Gestation*

	CD1 mice tested	Implantation site per mouse	Number of embryos that produced nitrate without LPS*	Number of embryos that produced nitrate with LPS*	Percent of embryos with significant nitrate production with LPS
Day 8	4	11.3 ± 2.2	1	2	4.4%
Day 10	4	12.8 ± 1.7	0	0	0.0%
Day 12	11	10.7 ± 3.3	2	5	4.3%

Cell from implantation sites of CD1 mice were treated in the same manner as those from CBA/J females as described in the legend for Table 1.
*Nitrate production above the upper 95% confidence limit (UCL 95%) established for day 12 nonresorbing embryos (>22 μM per 10^5 cells) was considered significant.

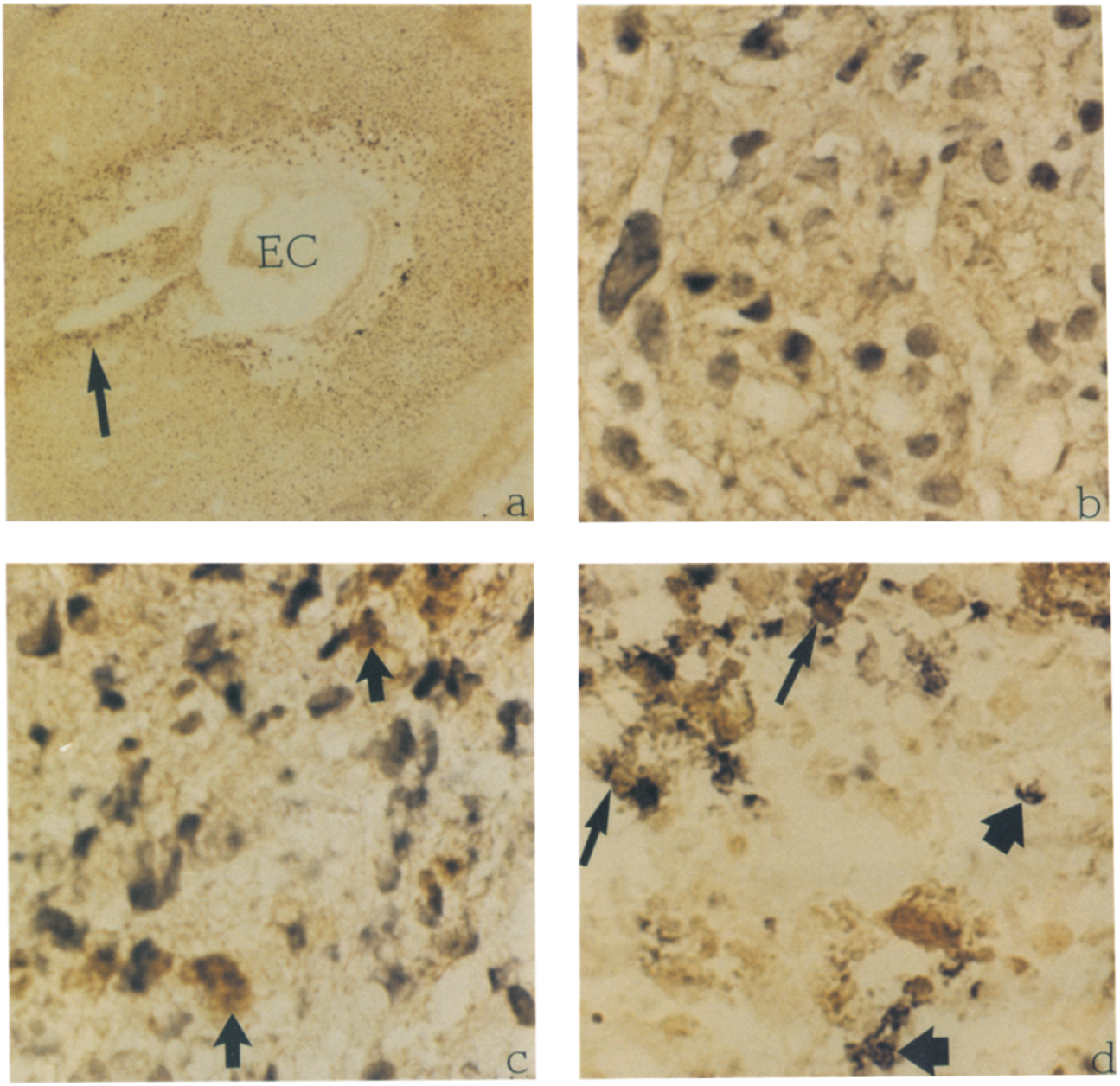

Figure 5. Detection of iNOS in mouse decidua. Frozen sections were air dried for 6 h, fixed in 2% paraformaldehyde for 30 min, and washed with Tris-HCl, pH 7.46, before staining. Sections were treated with (*a* and *c*) and without (*b*) anti-iNOS antibody, respectively, stained with DAB, and counterstained with Mayer's hematoxylin. (*a*) A low power view of iNOS staining in a day 8 embryo (×3.2). The embryonic capsule (*EC*) and the area of positive staining (*arrow*) are indicated. (*b*) A negative control stain in which the primary antibody was not added. (*c*) A population of iNOS-positive cells (*arrows*) surrounding the embryonic capsule (not shown) at ×600. (*d*) Sections were treated with both rabbit anti-iNOS and rat anti–Mac-1 antibodies at the same time. Horseradish peroxidase–conjugated goat anti–rabbit and alkaline phosphatase–conjugated goat anti–rat were then added. DAB (*brown*) and BCIP/NBT (*blue*) were substrates for the peroxidase and the phosphatase, respectively. Cells with single staining for Mac-1 (*large arrows*) and double staining (*thin arrows*) for Mac-1 and iNOS are indicated (×600).

decidua. At a magnification of 600, a population of iNOS-containing cells was seen around the embryonic capsule (Fig. 5 *c*). A negative control section with no primary antibody showed no significant staining (Fig. 5 *b*). To determine the cellular source of NO production by cells at the implantation sites, sections were stained with rabbit anti–mouse iNOS together with rat anti–Mac-1 antibodies. Fig. 5 *d* clearly shows a distinct population of double-stained cells. Some cells that stained only for Mac-1 were also seen in the same area.

Inhibition of NO Production Reduces Embryo Losses. To confirm the involvement of NO in early embryo loss, AG was used to inhibit the production of NO in vivo. AG was either injected or given orally in the drinking water to

Table 3. *AG-induced Reduction of Early Embryo Loss in CBA/J Female × DBA/2 Male Matings*

	Number of mice	Implantation sites per mouse	Viable embryos per mouse	Resorption rate (%)
Control mice PBS injection (i.p.)	5	8.0 ± 0.7	5.8 ± 1.0	27.1 ± 7.3
AG by injection (i.p.)	5	8.8 ± 2.4	8.0 ± 2.2	9.1 ± 6.0*
AG in drinking water	4	9.3 ± 1.5	8.5 ± 1.5	10.0 ± 5.7*

*p <0.05 compared to control. Using Student's t test, treated mice showed a significant decrease in resorption rates. CBA/J pregnant fremales were injected with 30 μg of polyI:C on day 6. From 6 until day 10, the mice were injected twice daily with 6 mg AG. Control mice received saline. A separate group of mice received AG (40 mM) in drinking water. The number of implantation sites together with the number of resorbing embryos were counted on day 12 of gestation.

Table 4. *AG-treated CBA/J × DBA/2 Matings Showed Increased Litter Size*

	Number of mice	Litter size
Control mice	10	6.3 ± 1.42*
AG-treated mice	10	8.1 ± 1.45*

*P < 0.05 compared to control using Student's t test. AG (40 mM) was provided in drinking water. AG-treated mice showed a significant increase in average litter size compared to control (mean ± SD). The litter were allowed to come to term, killed between 4 and 28 d after birth, and examined for gross morphological defects. No obvious morphological defects were seen in either group.

gravid CBA/J female mice starting on day 6 of gestation. In both cases, AG significantly reduced the resorption rate of CBA/J females mated with DBA/2 males (Table 3). This was determined by counting the number of dead embryos on day 12 of gestation. To show that AG increased embryo survival and litter size, AG-treated mice were allowed to go to term. Table 4 shows a significant increase in average litter size of AG-treated mice as compared to untreated controls. These results suggested that the production of NO by cells at the implantation sites was a major mediator of early embryo death.

Discussion

In this study, NO, a macrophage effector molecule, was shown to be involved in the mechanism associated with early embryo loss. Cells at the implantation sites from CBA/J and CD1 female mice were tested for nitrite and nitrate production by the Greiss reagent. On day 12 of gestation, resorbing embryos from both strains showed more than a fivefold increase in LPS-induced NO production compared to nonresorbing embryos. On the other hand, resorbing embryos were also shown to produce significant basal amounts of NO in the absence of LPS. This was not surprising, since on day 12, resorbing embryos were already damaged, and effector cells were assumed to be primed and activated. To demonstrate the involvement of NO in the mechanism of early embryo loss before pathologic changes in the embryo, we tested the production of nitrite and nitrate by placental cells on earlier days of gestation. Our results showed that the percentage of implantation sites from day 8–10 pregnant CBA/J and CD1 mice that were capable of producing NO corresponded to the natural incidence of resorption observed in both strains. Of note, we often detected considerably more nitrate than nitrite in these embryos. Similar preferential production of nitrate has been reported in mice infected with malaria (25). This may have been caused by the high oxidative capacity of the activated cells, which may have rapidly oxidized nitrite to nitrate. Furthermore, an increase in nitrite oxidation to nitrate by decidual cells could be one way of protecting embryos from the harmful effects of NO/NO_2^-. More interestingly, we detected an increased production of NO in the absence of LPS on days 8 and 10. This suggested that by day 8, the effector cells were already activated, and the damage may already have started.

The cellular source of NO production and hence the specific effector cells have not been fully explored in pregnant mice. NO can be constitutively produced by many cells, including vascular endothelial cells in placental tissues, and this may argue against an inducible NOS as the source of the observed NO production. However, our results showed that a more than fivefold increase in NO production by cells at implantation sites was induced by LPS, implicating the activation of the inducible NOS as a likely source of the NO produced. Furthermore, the placental cells from the resorbing embryos produced significantly more NO when challenged by LPS in vitro, further suggesting activated iNOS as the effector mechanism leading to embryo demise. Conversely, we were able to detect basal (constitutive) nitrite and nitrate production by nonresorbing placentas on day 12 and trace amounts on day 10 of gestation. This constitutive NO production may have been caused by the normal role that NO plays in uterine muscle relaxation (26). On the other hand, neutrophils can be induced to produce NO (27); however, their involvement in early embryo loss is unlikely because these cells were not notably present on days 8 and 10 of gestation (28). Never-

theless, using immunohistochemistry, we were able to detect a significant increase in F4/80 positive cells, a selective marker for macrophages, in resorbing compared to nonresorbing embryo (4). Furthermore, ~10% of the dispersed decidual cells expressed the Mac-1 and F4/80 markers. To further investigate the nature of the NOS involved in early embryo loss, we tested the presence of iNOS in frozen tissue sections of pregnant uteri by immunohistochemistry. These studies confirmed our observation of the involvement of a macrophage-associated inducible form of NOS in early embryo loss. In addition, uterine sections were also double stained for both iNOS and the macrophage marker Mac-1 (CD11b). As expected, iNOS-positive cells also stained with Mac-1, indicating decidual mononuclear cells as a likely cellular source of NO production. Some single-stained Mac-1–positive cells (iNOS negative) were also detected in day 8 decidua. Since few T cells or neutrophils have been observed in day 8 implantation sites, these cells may have been inactivated macrophages or NK cells (4). For these reasons, it appeared that in this study, a macrophage-associated iNOS was involved in the production of NO at the implantation sites.

To demonstrate the involvement of NO production in early embryo loss, the NOS inhibitor AG, was used to inhibit NO production in vivo. AG was shown to be a selective inhibitor for the iNOS, which was mainly present in activated macrophages, as opposed to the cNOS present in vascular endothelial cells and neurons (21–23). NO production was virtually completely abrogated when placental cells were incubated with AG in vitro (data not shown). More striking results were observed when mice were given AG in vivo. The resorption rate in CBA/J females was reduced from 28 to 11% when mice were either injected with AG or given AG in drinking water. These results directly showed the involvement of NO in the effector mechanisms of fetal rejection. When AG-treated mice were allowed to proceed to term, there was a significant increase in average litter size as compared to nontreated mice. The litters appeared morphologically normal when examined between birth and 4 wk of age. These studies showed that embryos that survive to day 12 of gestation usually survive to term without any significant additional losses because the number of viable embryos in each uterus on day 12 of gestation and the number of viable pups at delivery, were not statistically different.

The mechanisms of early fetal rejection are regulated by a complex network of cytokines. It is known that only primed and not resting macrophages produce NO when challenged with either LPS or TNF-α (29). IFN-γ primed macrophages in vivo as well as in vitro (30). Our data showed that placental mononuclear cells in potentially resorbing embryos were primed and could produce NO when challenged with LPS in vitro. The source of the priming molecule could be IFN-γ or TNF-α, which may be produced by infiltrating NK cells. In fact, injecting gravid mice with IFN-γ or poly I:C, an activator of IFN production, was shown to increase the resorption rate in CBA/J females when mated with DBA/2 males (2). Poly I:C could therefore have indirectly increased decidual macrophage priming by inducing IFN-γ and/or TNF-α production by decidual NK cells, and hence an increase in NO production. Furthermore, the association of NK cells with early embryo loss has been observed (5). TNF-α, which is also produced by activated macrophages, was associated with embryo resorption in CBA/J × DBA/2 mice (6, 10). We have previously reported the production and the release of TNF-α within the pregnant uterus and that embryo loss was reduced by the TNF-α inhibitor pentoxifylline (6). Similarly, others showed that inoculation of gravid mice with recombinant TNF-α induced early embryo loss (10). The presence at the implantation sites of both IFN-γ and TNF-α, which cooperatively promote NO production by macrophages, implies that a macrophage-inducible NOS is involved in early embryo loss.

The results in this work demonstrate the involvement of NO in early embryo loss and implicates the activation of primed decidual macrophages in effecting spontaneous abortion.

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