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Mammalian nitrate biosynthesis: Mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide

(nitrosamines/lymphokines/carcinogenesis)

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ABSTRACT **_Escherichia coli_ lipopolysaccharide (LPS)-induced nitrate biosynthesis was studied in LPS-sensitive C3H/He and LPS-resistant C3H/HeJ mice. Intraperitoneal injection of 15 μg of LPS led to a temporary 5- to 6-fold increase in blood nitrate concentration in the C3H/He strain. Levels of nitrate excreted in the urine were also increased. In contrast, no increase was observed in the C3H/HeJ strain with LPS injections up to 175 μg. Furthermore, thioglycolate-elicited peritoneal macrophages from C3H/He, but not from C3H/HeJ mice, produced nitrite (60%) and nitrate (40%) when cultured with LPS (10 μg/ml). T-lymphocyte addition/depletion experiments showed the presence of T cells enhanced this response. However, LPS did not cause nitrite or nitrate production in cultures of spleen lymphocytes from either strain. LPS-induced nitrate synthesis was also observed with nude mice and CBA/N mice, indicating that neither functional T lymphocytes nor LPS-responsive B lymphocytes were required for the response _in vivo_. This was consistent with the _in vitro_ results showing macrophages alone were competent. _Mycobacterium bovis_ infection of C3H/He and C3H/HeJ mice resulted in a large increase in nitrate production over the course of the infection for both strains, suggesting T-lymphocyte-mediated activation of macrophages as a potent stimulus for nitrate biosynthesis. The synthesis of nitrite is significant in that it can directly participate in the endogenous formation of nitrosamines and may also be involved in some aspect of the chemistry of cytotoxicity.**

A number of studies have now established that mammals on low nitrate diets excrete more nitrate than they ingest (1–3). At first it was thought that this excess urinary nitrate was the product of intestinal microbial metabolism (1), but subsequent studies with germ-free and conventional rats showed that the process of nitrate biosynthesis was by a mammalian pathway (2). The concern for this process stems from the demonstrated carcinogenicity of nitrosamines (4), the association between exposure to high levels of nitrate (NO_3^-) and stomach cancer (5–8), and the potential for the endogenous formation of nitrosamines by reaction of nitrite derived from nitrate by bacterial reduction (9–11) with endogenous or exogenous amines.

Tannenbaum and colleagues (12) have shown that treatment of rats with *Escherichia coli* lipopolysaccharide (LPS) led to approximately a 10-fold increase in urinary NO_3^-. They also showed through the use of ^{15}N-labeled ammonia that ammonia was incorporated into NO_3^- (12). This finding was supported by subsequent studies, which included *in vitro* experiments, that suggested the involvement of oxygen radicals in the oxidation (13, 14). The results of Tannenbaum and coworkers mentioned above provided an approach to the questions of the cell types and biochemical processes involved in this NO_3^- biosynthesis. Through experiments with cells in culture and a LPS-stimulated mouse model that utilized several mouse strains that exhibit specific genetic immunocellular defects, we have found that macrophages are one, and possibly the major, source of urinary NO_3^- in LPS-treated mice.

MATERIALS AND METHODS

Animals. The following strains of mice were used: C3H/HeJ (The Jackson Laboratory); C3H/He, BALB/c *nu/nu*, BALB/c *nu/+* (Charles River Breeding Laboratories); CBA/NCr, CBA/JCr (Frederick Cancer Research Facility, Frederick, MD). All mice used were 9–12 weeks old except for the BALB/c and CBA mice, which were 7 and 5 weeks old, respectively.

Housing and Urine Collection. Mice were housed three to a cage in Nalgene metabolic cages (Thomas) and were given a nutritionally complete low-nitrate diet (12) and distilled drinking water. The urine from three mice was automatically pooled and collected every 24 hr. The cages were cleaned daily with germicidal soap. In all cases, the amount of nitrate in the urine after a 24-hr collection period for three mice was divided by three and reported as the mean daily nitrate production per mouse. As an additional precaution against infection, nude mice were housed in an isolated cubicle, given sterile water, and transferred daily with sterile holders into autoclaved metabolic cages at the time of urine collection.

Blood Collection. Blood was obtained by heart puncture under ether anesthesia with heparinized syringes. The blood samples were protein-precipitated (30% $ZnSO_4$, 0.05 ml per ml of blood), centrifuged, and filtered (pore diameter, 0.22 μm) prior to analysis.

Nitrite and Nitrate Analysis. Nitrate and nitrite in blood, urine, and cell culture supernatant samples were measured by an automated procedure (15). Briefly, samples are passed through a column containing copper-plated cadmium filings, thereby reducing sample nitrate to nitrite. The sample then undergoes reaction with the Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) to form a chromophore absorbing at 543 nm. Nitrite can be measured independently of nitrate by bypassing the cadmium column.

Administration of Calmette–Guerin Bacillus (BCG) and LPS. BCG (ATCC 19274) was grown in Dubos medium (16). Twelve- to fourteen-day cultures were used for injections. The bacterial culture was centrifuged at 10,000 × *g* and the bacteria were resuspended in sterile saline prior to injection. The number of viable units injected was determined by plate count on Middleton 7H10 agar (Interstate Media). Sterile

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Abbreviations: LPS, lipopolysaccharide; BCG, *Mycobacterium bovis* (Calmette–Guerin bacillus); PEC, peritoneal exudate cells.

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stock solutions of *E. coli* LPS (serotype 0127:B8; Sigma) of 100 or 250 mg/ml were prepared and diluted in saline prior to intraperitoneal injections.

Cell Collection and Culture. Thioglycolate-elicited peritoneal macrophages were obtained from C3H/He and C3H/HeJ mice by injection of 1.0 ml of sterile thioglycolate solution 4–8 days prior to lavage with 10 ml of Hanks' buffered saline solution. The peritoneal exudate cells (PEC) were centrifuged at 200 × *g* and washed once with Dulbecco's modified Eagle's medium (GIBCO) supplemented with 40 mM glutamine/5% endotoxin-free heat-inactivated fetal calf serum (HyClone, Logan, UT)/penicillin (5 international units/ml)/streptomycin (5 μg/ml) (DMEM-5). The cells were resuspended in DMEM-5 to 2–8 × 10^6 cells per ml. Viability was >95% as determined by trypan blue dye exclusion. The PEC were plated (1.5 ml of cell suspension onto 35-mm plates) and incubated for 1 hr at 37°C in 5% CO_2/95% air to allow for macrophage adherence. The plates were then washed once with warm DMEM-5 to remove nonadherent cells and the appropriate experimental medium (DMEM-5 ± LPS at 10 mg/ml) was added. This procedure resulted in an adherent cell population that was 80–90% macrophages, as determined by morphology and esterase staining.

In some cases, a portion of the PEC suspension was depleted of T lymphocytes by first adding mouse anti-Thy-1.2 monoclonal antibody (1:50 dilution, 4°C, 30 min) followed by addition of rabbit (1:50 dilution) and guinea pig (1:10 dilution) complement containing serum. The treated cell suspension was then plated as described above and incubated for 1 hr to allow T-cell lysis and macrophage adherence to occur. The plates were then washed with DMEM-5 and the experimental medium was added.

Spleen cells were prepared as described (17). Briefly, spleens from six C3H/He mice were teased apart by using forceps and a cotton swab. The suspension was filtered through plastic gauze, centrifuged at 400 × *g*, and washed once with DMEM-5 and plated (15 ml per plate) onto two 100-mm tissue culture plates. After incubation for 1 hr at 37°C, the nonadherent spleen cells were poured off and saved.

Nonadherent spleen cells were added to macrophage cultures at 5.5 × 10^6 cells per plate in 0.5 ml of DMEM-5. A portion of the spleen cells was recentrifuged at 400 × *g*, diluted to 6 × 10^6 cells per ml in experimental medium (DMEM-5 ± LPS at 10 μg/ml), and plated at 2.0 ml per plate.

All experimental cultures of macrophages and spleen cells were incubated for 24 hr at 37°C in 5% CO_2/95% air. After 24 hr, the culture supernatants were collected and stored at −10°C until analysis. Phenol red was found to interfere with the NO_2^-/NO_3^- determination and was removed by passing the culture supernatant samples through Sep Pak C_{18} columns (Waters Associates) before analysis.

Protein Determination. After supernatant collection, the plated cells were washed twice with 2.0 ml of Hanks' buffered saline solution. The wash fluid was removed, 1.0 ml of 1% Triton X-100 solution was added, and the cells were incubated for 30 min at 35°C. The plates were then scraped with a rubber policeman and the protein content of each suspension was determined by the Bradford assay (18) using plates that contained medium but no cells as controls.

RESULTS

Fig. 1*A* illustrates how the concentration of NO_3^- in the blood changes after an intraperitoneal injection of 15 μg of LPS in both LPS-responsive (C3H/He) and nonresponsive (C3H/HeJ) mice. In C3H/He mice, there was a 2- to 4-hr lag period followed by a rapid increase in blood NO_3^- concentration. The peak NO_3^- level was observed between 8 and 12 hr after injection in the C3H/He mice. At 10 hr postinjection, the level of blood NO_3^- was increased 5 to 6 times that of the average basal level. In contrast, C3H/HeJ mice receiving LPS showed no increase in blood NO_3^- level after LPS injection. When this experiment was repeated using female mice of both strains, similar results were obtained (data not shown).

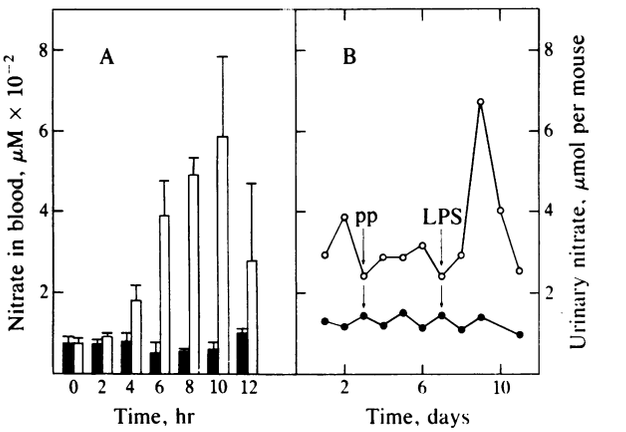

FIG. 1. (*A*) NO_3^- levels in blood after intraperitoneal injection of LPS (10 μg) into C3H/He (□) and C3H/HeJ (■) mice. (*B*) Daily urinary NO_3^- excretion for C3H/He (○) and C3H/HeJ (●) mice both before and after intraperitoneal injections of proteose peptone (pp) on day 3 and 20 μg of LPS on day 7. In *A*, each point represents the value per mouse ± SEM averaged for three mice. In *B*, each point represents the daily value per mouse averaged for three mice.

Fig. 1*B* shows how total NO_3^- production (as measured by NO_3^- excreted in urine) in C3H/He and C3H/HeJ mice is affected after i.p. injections of a sterile eliciting agent (proteose peptone) and 20 μg of LPS. Injection of 1 ml of 4% proteose peptone solution did not alter subsequent NO_3^- production in either C3H/He or C3H/HeJ mice. An injection of 20 μg of LPS caused an increase in NO_3^- excretion in the C3H/He but not in C3H/HeJ mice. NO_3^- excretion is shown to have peaked during the 24- to 48-hr time block following LPS injection. This was not always the case. In other experiments, the greatest quantity of NO_3^- was sometimes excreted in the first 24 hr after injection. Fig. 1*B* shows that NO_3^- excretion was increased to 2.5 times the normal basal level on the peak day (day 9) and then returned to basal level on the following days. Mice receiving only the 20-μg LPS injection and not proteose peptone had responses that were identical to the proteose peptone pretreated mice both in terms of their response kinetics and in magnitude of increase in NO_3^- production (data not shown). Also, NO_3^- production in control C3H/He and C3H/HeJ mice receiving injections of sterile saline remained at basal levels in the days following injection.

To further test the refractory response of C3H/HeJ mice to LPS, several groups of C3H/HeJ mice were injected with larger amounts of LPS. C3H/HeJ mice did not excrete increased levels of NO_3^- in response to LPS even at the highest dose tested (175 μg per mouse).

BCG infection is known to hypersensitize mice to LPS (19). It has also been shown to sensitize C3H/HeJ mice so they respond to doses of LPS that would normally be too low to be effective (20). Fig. 2 *A* and *B* shows that when C3H/HeJ and C3H/He mice were infected with BCG, a very large increase in NO_3^- production occurred over the course of the infection. Both strains of mice maintained basal levels of NO_3^- excretion for 4–5 days after BCG injection. On the 6th day after infection, NO_3^- excretion began to increase rapidly in both strains and reached a peak of ≈90 μmol per day on the 10th day after injection. A sharp decline in daily NO_3^- excretion followed.

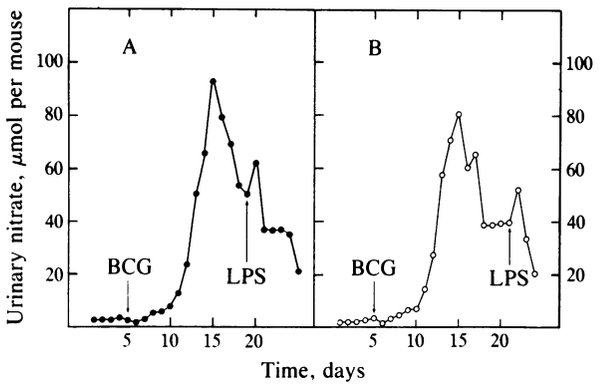

FIG. 2. NO_3^- in urine excreted by C3H/HeJ (*A*) and C3H/He (*B*) mice both before and after BCG infection (day 5) and i.p. injection of LPS. C3H/HeJ mice received 75 μg of LPS on day 19; C3H/He mice received 15 μg of LPS on day 21. Each point represents the daily value per mouse averaged for three mice.

The magnitude of the BCG-induced increase in NO_3^- excretion was dependent on the number of infectious units of BCG each mouse received (data not shown). When the mice received fewer infectious units, there was a corresponding decrease in the amount of NO_3^- excreted over the course of the infection. However, the peak excretion level was reached at the same time (10–12 days after infection), irrespective of the number of infectious units injected. Also, viable cultures of BCG in Dubos medium, ranging in age from 3 to 25 days, did not contain detectable levels of NO_3^-.

Fig. 2*A* also shows that when LPS (75 μg) was injected into the BCG-infected C3H/HeJ mice on day 19, they responded with an increase in NO_3^- excretion of 12 μmol/day in the 24 hr following LPS injection. This increase is significant when compared to a group of identically treated BCG-infected C3H/HeJ mice that did not receive LPS on day 19. The mice that did not receive the LPS showed a decrease in NO_3^- production of 9.5 μmol/day in the same 24-hr period (data not shown). The experiment was repeated by injecting 175 μg of LPS into a separate group of BCG-infected C3H/HeJ mice on day 21. This also resulted in an increase in NO_3^- synthesis of 11 μmol/day in the 24-hr period following LPS injection (data not shown). A similar result was seen with BCG-infected C3H/He mice (Fig. 2*B*). A 15-μg injection of LPS into these mice on day 21 caused an increase in NO_3^- excretion of 12 μmol/day in the 24 hr following the LPS injection. This dose of LPS normally caused an increase in NO_3^- excretion of 4 μmol/day in non-BCG-infected C3H/He mice.

Mice lacking a functional population of T cells (nude mice) were used to determine whether the presence of T cells was necessary for LPS-induced nitrate biosynthesis to occur. The results are summarized in Table 1. Nude mice given 40 μg of LPS excreted increased amounts of NO_3^-. Increased NO_3^- excretion in nude mice peaked during the second 24-hr period after LPS injection. The increases in NO_3^- production following LPS injection were in accord with those seen in closely related *nu*/+ mice (Table 1).

Table 1. LPS-induced nitrate production in T-cell- and B-cell-deficient mice

Strain	Basal*	LPS-induced†
CBA/N	1.3 ± 0.1	3.1
CBA/J	0.9 ± 0.1	4.2
nu/nu	1.5 ± 0.3	11.9
nu/+	1.5 ± 0.2	3.3

*The amount of urinary nitrate excreted daily (μmol per mouse) averaged over several days for three mice.

†The average urinary nitrate (μmol per mouse) excreted in excess of the mean basal levels for three mice and summed over the 3 days after a 40-μg LPS injection.

We also investigated whether B-lymphocyte responses to LPS play a role in the LPS-induced nitrate response by using CBA/N mice. These mice have an X-linked defect in B-lymphocyte function, making them unable to form antibody to protein-free LPS (21, 22). CBA/N B cells also have a greatly diminished mitogenic response to LPS *in vitro* (23). The basal and LPS-induced NO_3^- production in CBA/N mice was compared to that of the closely related CBA/J strain, which has been shown to possess LPS-responsive B lymphocytes (24). The results of these experiments are also shown in Table 1. Injection of 40 μg of LPS resulted in increased NO_3^- excretion in both strains, with peak nitrate excretion occurring either in the first or second 24-hr time period following LPS injection. CBA/J mice exhibited similar increases in NO_3^- production when injected with the same dose of LPS.

We next performed *in vitro* experiments with cultures of peritoneal macrophages to see if these cells would synthesize NO_3^- in response to LPS. Cultures of thioglycolate-elicited macrophages from C3H/He and C3H/HeJ mice were treated with LPS (10 μg/ml) for 24 hr and the final culture supernatant NO_2^- and NO_3^- concentrations were compared to those of control cultures not receiving LPS. Table 2 shows that macrophages from C3H/He mice cultured with LPS produced levels of both NO_2^- and NO_3^- greater than in control cultures not exposed to LPS. In contrast, C3H/HeJ macrophages cultured with LPS did not produce increased levels of NO_2^-/NO_3^- compared to control cultures. In all cases, NO_2^- represented ≈60% of the total NO_2^-/NO_3^- present at the time of analysis.

We next tested the effect of T-lymphocyte depletion or spleen lymphocyte addition on LPS-induced NO_2^-/NO_3^- synthesis in cultures of C3H/He macrophages (Table 2). LPS-induced NO_2^-/NO_3^- production in C3H/He macrophage cultures depleted of T lymphocytes was less than that of identically treated control macrophage cultures (17.6 nmol per 10^6 cells vs. 30.0 nmol per 10^6 cells, respectively), which in turn was less than that of macrophage cultures that had had nonadherent spleen lymphocytes added (56.2 nmol per 10^6 cells).

The total protein remaining on each plate was measured at the conclusion of the lymphocyte addition/depletion experiment (Table 2). Macrophage cultures treated with LPS that had been depleted of lymphocytes had an average protein content of 365 ± 30 μg per plate, whereas control macrophage cultures receiving LPS had an average protein content of 308 ± 95 μg per plate. Macrophage cultures receiving both LPS and spleen lymphocytes had an average protein content of 595 ± 26 μg per plate.

To determine if LPS had caused added or residual lymphocytes in the macrophage cultures to produce NO_2^-/NO_3^-, nonadherent spleen lymphocytes were cultured with LPS (10 μg/ml) for 24 hr and NO_2^-/NO_3^- production was assayed. LPS did not cause NO_2^- or NO_3^- production by spleen lymphocytes under these conditions (Table 2).

DISCUSSION

The results presented clearly show that thioglycolate-elicited C3H/He macrophages produce NO_2^-/NO_3^- when cultured with LPS. T lymphocytes appear to amplify NO_2^-/NO_3^- production in culture. Also, the results indicate that the macrophage is most likely the primary cell involved in LPS-induced NO_2^-/NO_3^- synthesis *in vivo*.

The C3H/HeJ mouse carries a mutation that renders it hyporesponsive to LPS (25, 26). Through studies with both C3H/HeJ and the LPS-responsive C3H/He strain, others have shown that the cells responsible for the biological

Table 2. Nitrite and nitrate production by macrophage cultures

Contents	Final concentration NO_3^-/NO_2^-, μM	Final concentration NO_2^-, μM	NO_3^-/NO_2^-, nmol per 10^6 cells*	Protein, μg per plate
C3H/He	17.4 ± 0.4	8.3 ± 1.9	0.2	243 ± 9
C3H/He ± LPS	152.7 ± 1.8	91.3 ± 4.9	30.0	308 ± 95
C3H/HeJ	15.6 ± 1.2			
C3H/HeJ + LPS	15.6 ± 1.1			
C3H/He + AB/C†	17.8 ± 0.7	6.8 ± 0.4		405 ± 18
C3H/He + AB/C† + LPS	96.3 ± 7.0	47.9 ± 2.8	17.6	365 ± 30
C3H/He + Sp cells‡	19.6 ± 2.8	7.3 ± 0.7		607 ± 84
C3H/He + Sp cells‡ + LPS	271.2 ± 4.9	111.1 ± 1.8	56.2	595 ± 26
Sp cells§	16.1 ± 0.7	8.1 ± 0.4		
Sp cells§ + LPS	15.6 ± 0.5	7.0 ± 0.3		
Medium + LPS	16.5 ± 0.4	0.0 ± 0.0	0	

Thioglycolate-elicited C3H/He or C3H/HeJ macrophages were incubated with or without LPS (10 μg/ml) for 24 hr. NO_2^-/NO_3^- concentration values represent the levels present in the supernatant after a 24-hr incubation using equal numbers of macrophages and equal volumes. Protein values are the adherent cell protein present after 24 hr and supernatant removal. Each value in the table represents the mean ± SEM of three cultures.

*Derived by dividing the NO_2^-/NO_3^- present (in nmol) in each 24-hr culture supernatant (minus the background NO_2^-/NO_3^- in the medium) by the number of PEC plated per dish ($\times 10^{-6}$) as described in *Materials and Methods*.

†Macrophage cultures were depleted of T cells by antibody and complement lysis.

‡C3H/He spleen (Sp) cells (5.5×10^6) were added to each macrophage culture at the start of the experiment.

§C3H/He spleen cells (12×10^6 per plate) were cultured with and without LPS (10 μg/ml) for 24 hr.

effects of LPS are of lymphoreticular origin (27). We have found (Fig. 1 *A* and *B*) that LPS caused increased NO_3^- production in C3H/He mice but not in C3H/HeJ mice, indicating that lymphoreticular cells were involved in LPS-induced NO_3^- biosynthesis.

BCG infection renders LPS-nonresponsive C3H/HeJ mice almost as sensitive to LPS as normal mice (20). The cellular basis for this effect was shown to be T-lymphocyte regulation of macrophage sensitivity to LPS (28). Therefore, it was of interest to see if BCG infection would also enable the C3H/HeJ mouse to produce NO_3^- in response to LPS. Our results (Fig. 2*A*) show that C3H/HeJ mice, whose macrophages had been rendered responsive to LPS from BCG infection, were now able to produce increased amounts of NO_3^- in response to LPS. This suggested macrophage involvement in NO_3^- biosynthesis.

We also used the CBA/N mouse strain to determine whether any LPS-induced B-lymphocyte responses were involved in NO_3^- biosynthesis. LPS is a B-cell mitogen (29), induces antibody synthesis (21), and triggers B-lymphokine secretion (30). However, our data show that B-cell responses to LPS are not involved in LPS-induced NO_3^- biosynthesis.

Our next experiments with nude mice focused on the role of the T lymphocyte in LPS-induced NO_3^- biosynthesis. Nude mice have been shown to have a normal or slightly amplified response to LPS both *in vivo* (31) and by their macrophages in culture (32, 33). Our results showed that the absence of T cells did not affect either basal or LPS-induced NO_3^- synthesis in nude mice, suggesting that T cells are not required for LPS-induced NO_3^- biosynthesis.

The above results are consistent with the macrophage as the cell type involved in LPS-induced NO_3^- biosynthesis. Our *in vitro* experiments involving plated macrophages show that thioglycolate-elicited C3H/He macrophages synthesized NO_2^-/NO_3^- when cultured with LPS. However, C3H/HeJ macrophages when treated analogously did not synthesize either NO_2^- or NO_3^-.

The T-cell depletion experiments (Table 2) indicate that macrophages are producing NO_2^-/NO_3^- directly in response to LPS without the involvement of T cells. These results, along with our preliminary finding that the macrophage-like tumor cell line J774.1 is capable of producing NO_2^-/NO_3^- in response to LPS (unpublished observation), shows NO_2^-/NO_3^- synthesis is due to a direct effect of LPS on macrophages and does not require the presence of another cell type. However, it appears that if residual or added T cells are present, there is an increase in the amount of NO_2^-/NO_3^- produced. Nonadherent spleen cells incubated with LPS did not produce NO_2^-/NO_3^-, indicating that the increased production in the macrophage cultures containing either residual or added lymphocytes was not due to LPS-induced lymphocyte NO_2^-/NO_3^- synthesis. One possible interpretation of these results is that T lymphocytes, when present, further activate the macrophage (34), causing an amplification in LPS-induced macrophage NO_2^-/NO_3^- synthesis.

The results of the BCG experiment further support that T lymphocytes may play a role in NO_3^- biosynthesis under certain circumstances. BCG infection causes a pronounced activation of the host animal's macrophages (34, 35). Certain T-cell lymphokines are known to activate (36, 37) and thought to mediate the activation of macrophages during BCG infection (28). It is possible that the NO_3^- generated during BCG infection is produced by macrophages that have been activated by T-cell lymphokines. The observed 5- to 6-day delay in BCG-induced NO_3^- production is consistent with T-cell involvement and does not support a direct activation of macrophage NO_3^- synthesis by BCG. However, at this point we cannot rule out NO_2^-/NO_3^- production by other cells during infection.

The time course of NO_3^- production during BCG infection parallels other immunological changes that occur, such as development of nonspecific bacterial resistance (35, 38), macrophage activation to a microbicidal and tumoricidal state (34, 39), and lymphocyte proliferation (40). This suggests that the magnitude of NO_3^- biosynthesis may be directly related to the state of activation of the immune system. The fact that BCG infection caused an equal amplification in NO_3^- production in both C3H/He and C3H/HeJ mice is interesting in that BCG infection has been shown to activate the macrophages of these two strains to different degrees in terms of tumor cytotoxicity (41).

It appears that macrophages need to achieve a certain level of activation before producing NO_2^-/NO_3^-. Macrophages elicited with thioglycolate, although activated with respect to

resident macrophages (42, 43), produce no detectable NO_2^-/NO_3^- *in vitro*. Also, animals receiving proteose peptone injections did not produce increased amounts of NO_3^-. However, macrophages exposed to LPS in culture, or possibly activated during BCG infection, do produce increased amounts of NO_2^-/NO_3^-. Although this indicates that macrophages engage in NO_3^- production during immunostimulation, their role in NO_3^- biosynthesis during basal conditions is an open question. Increased nitrate synthesis by macrophages treated with LPS or BCG corresponds with increased production of interleukin 1, prostaglandin E_2, and colony-stimulating activity (36, 44). It remains to be seen whether the secretion of these monokines and production of NO_2^-/NO_3^- are related in some way.

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