Effects of *Mycobacterium bovis* BCG Infection on Regulation of L-Arginine Uptake and Synthesis of Reactive Nitrogen Intermediates in J774.1 Murine Macrophages

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The generation of nitric oxide (NO) by activated macrophages is believed to control mycobacterial infection in the murine system. In this study we examined the effect of *Mycobacterium bovis* BCG infection on the L-arginine-dependent NO pathway in J774.1 murine macrophages. We have confirmed previous results by demonstrating that stimulation of J774.1 with lipopolysaccharide (LPS) and gamma interferon (IFN-γ) results in an increase in the uptake of ^{3}H-labeled L-arginine and a concomitant increase in the production of NO. We have also shown that BCG can mimic LPS treatment, leading to enhanced L-[^{3}H]arginine uptake by IFN-γ-stimulated macrophages. Lipoarabinomannan, a component of the BCG cell wall that is structurally similar to LPS, is not responsible for the uptake stimulation in IFN-γ stimulated macrophages. Although we demonstrated that there was a 2.5-fold increase in NO production by macrophages 4 h after LPS–IFN-γ stimulation, BCG infection (with or without IFN-γ stimulation) did not lead to the production of NO by the macrophages by 4 h postinfection. At 24 h postinfection, the infected macrophages that were stimulated with IFN-γ produced amounts of NO similar to those of macrophages stimulated with LPS–IFN-γ. This suggests that there are multiple regulatory pathways involved in the production of NO. Finally, our data suggest that increased expression of the arginine permease, MCAT2B, after 4 h of LPS–IFN-γ treatment or BCG infection–IFN-γ treatment is not sufficient to account for the increases in L-[^{3}H]arginine uptake detected. This suggests that the activity of the L-arginine transporter(s) is also altered in response to macrophage activation.

The successful replication and survival of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, within the phagosomes of human alveolar macrophages is partially attributed to the ability of the bacterium to evade many of the antimicrobial activities established by the activated macrophages. Some of these activities include acidification of the phagosomal compartment, the production of reactive oxygen intermediates, and the production of reactive nitrogen intermediates. Mycobacteria interfere with several steps of the macrophage phagosomal-lysosomal maturation pathway, including the exclusion of proton ATPases from the lysosomal membrane. This prevents the acidification of the compartment in which the mycobacteria reside, which in turn inhibits the activation of pH-dependent lysosomal degradative enzymes (29). Several studies have shown that mycobacteria may not be susceptible to the toxic effects of reactive oxygen intermediates due to the presence of mycobacterial compounds such as glycolipids, sulfatides, and lipoarabinomannans (5, 6, 23). In contrast, the generation of nitric oxide (NO) by activated macrophages is believed to be involved in the control of mycobacterial infection in the murine system (7). The antimycobacterial effects of NO production are mediated by excessive lipid peroxidation of sulfhydryls, tyrosine residues, heme and non-heme irons, and iron-sulfur proteins and centers (reviewed in reference 27).

The synthesis of NO by macrophages is induced upon stimulation of the macrophage with bacterial lipopolysaccharide (LPS) and/or cytokines such as gamma interferon (IFN-γ) (11, 28). NO is generated from L-arginine by the inducible, Ca^{+2}-calmodulin-independent, and NADPH-dependent nitric oxide synthase (iNOS). The sustained synthesis of high levels of NO by activated macrophages is to a great extent dependent upon the presence of extracellular L-arginine (13). In fact, extracellular L-arginine is used preferentially to the L-arginine present in the intracellular pools to generate NO by activated macrophages (2, 14). Several studies have demonstrated that LPS and IFN-γ induce a time-dependent stimulation of L-arginine transport activity in cultured J774 murine macrophages to supply extracellular L-arginine for the production of NO (2, 4). Stimulation of L-arginine transport and the production of NO can be detected as early as 4 h following stimulation of murine macrophages with LPS and IFN-γ (2, 4).

There are three known proteins responsible for the transport of extracellular L-arginine in the murine system: MCAT1, MCAT2A, and MCAT2B (9, 10, 19). All three transporters are cationic amino acid transporters, responsible for the transport of L-arginine, L-lysine, and L-ornithine. These transporters belong to the mammalian high-affinity y$^+$ family of transporters that are Na$^+$-independent, pH-independent, and subject to trans-stimulation. MCAT1 transcripts are for the most part constitutively expressed in normal tissues and cell lines (21). This transporter is responsible for the uptake of L-arginine for basic macrophage metabolism. Interestingly, Kakuda et al. (16) have shown that the MCAT1 mRNA levels decreased 24 h after LPS–IFN-γ stimulation.

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The generation of MCAT2A or MCAT2B occurs by alternative splicing of a single mRNA transcript. MCAT2A is exclusively detected in hepatocytes, replacing MCAT1 (9). The mRNA expression and, subsequently, the protein expression of MCAT2B are induced upon LPS–IFN-γ stimulation of murine macrophages (10, 16). The 5′ untranslated region of MCAT2B contains five distinct promoter regions. The most distal promoter (approximately 18 kb upstream from the first coding exon) is the promoter responsive to macrophage activation by LPS–IFN-γ stimulation (12). Using *Xenopus* oocytes, Kakuda et al. (17) were able to demonstrate that MCAT2B was solely responsible for the increased uptake of L-arginine detected upon LPS–IFN-γ stimulation of the macrophage.

The aim of these studies was to examine the effect of *M. bovis* BCG infection on the L-arginine dependent NO pathway in J774.1 murine macrophages. We demonstrate that BCG can mimic LPS leading to enhanced L-[³H]arginine uptake by IFN-γ-stimulated macrophages and that there are multiple regulatory pathways involved in the production of NO. Our data also suggest that the kinetics of L-arginine transport are altered in response to macrophage activation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. bovis*-BCG Pasteur strain (Difco) was grown in Middlebrook medium [per liter: (NH₄)SO₄, 0.5 g; L-glutamic acid, 0.5 g; sodium citrate, 0.1 g; pyridoxine, 0.001 g; biotin, 0.0005 g; Na₂HPO₄, 2.5 g; KH₂PO₄, 1.0 g; ferric ammonium citrate, 0.04 g; MgSO₄, 0.05 g; CaCl₂, 0.0005 g; ZnSO₄, 0.001 g; CuSO₄, 0.001 g]. Middlebrook 7H9 (liquid) and 7H11 (1.5% agar) media (Difco) were supplemented with glycerol (0.5%, vol/vol) and ADC supplement (0.5% bovine serum albumin, fraction V [Roche Pharmaceuticals], 0.2% dextrose, 0.85% NaCl). All liquid cultures of BCG were supplemented with 0.05% Tween 80 (Sigma).

Macrophage culture. J774.1 cells were maintained in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Sigma), amino acids (BioWhittaker), and L-glutamine (Sigma).

Incubation of cells with LPS, IFN-γ, or both. J774.1 cells were subcultured into 96-well tissue culture plates at a cell density of 1.5×10^5 cells/well and incubated at 37°C in a 5% CO₂ for 2 h to allow the cells to adhere. The adherent J774.1 macrophages were then incubated for 4 or 24 h (where indicated) with either 1 μg of LPS (Sigma) per ml, 100 U of IFN-γ (Gibco-BRL) per ml, or both. The concentrations of LPS and IFN-γ used for these studies are the concentrations that led to optimal L-arginine transport stimulation and NO production in J774.1 macrophages (4). Where appropriate, the macrophages were treated with 1.0 mg of cycloheximide (Sigma) for 1 h following stimulation to block protein synthesis. To ensure that protein synthesis was blocked, a trichloroacetic acid (TCA) precipitation was performed on macrophages exposed to 100 μM L-[³H]arginine for 10 min. No TCA-precipitable counts were detected.

BCG infection of J774.1 macrophages. Mid-logarithmic ($A_{600} = 0.35$ to 0.55) BCG were washed three times in DMEM with vigorous vortexing to remove any mycobacterial clumps. Following the third wash, the BCG was resuspended in 10 ml of DMEM with or without 100 U of IFN-γ per ml, and the remaining mycobacterial clumps were removed by passing the mycobacterial suspensions through a 5-μm (pore-size) filter. To initiate the infection, 200 μl of the BCG suspensions (multiplicity of infection of 5) was added to the adherent macrophages in the 96-well plates and incubated for 4 or 24 h was as indicated.

Infection of J774.1 with heat-killed BCG or *M. tuberculosis* H37Rv. Mid-logarithmic BCG or *M. tuberculosis* were heat killed at 60°C for 10 min. Following inactivation, the mycobacteria were processed the same as for the live bacteria (see above) and used to infect adherent J774.1 macrophages for 4 h.

Incubation of IFN-γ costimulated macrophages with BCG and *M. tuberculosis* LAM. J774.1 cells were subcultured into 96-well tissue culture plates at a cell density of 1.5×10^5 cells/well and incubated at 37°C in 5% CO₂ for 2 h to allow the cells to adhere. The adherent J774.1 macrophages were then incubated for 4 h with 100 U of IFN-γ (Gibco-BRL) per ml and either 10 μg of BCG lipoarabinomannan (LAM) or 10 μg of *M. tuberculosis* Erdman LAM per ml (kindly supplied by John Belisle). The concentrations of BCG and *M. tuberculosis* Erdman LAM used for these studies are the concentrations of LAM leading to

optimal NO production by activated macrophages (Edward Chan, unpublished data).

Measurement of L-arginine and L-proline transport. According to a protocol developed by Bogle et al. (4), 1.5×10^5 adherent macrophages in 96-well plates were rinsed three times in prewarmed HEPES-buffered Krebs solution (NaCl, 131 mM; KCl, 5.5 mM; MgCl₂, 1.0 mM; CaCl₂, 2.5 mM; NaHCO₃, 25 mM; NaH₂PO₄, 1.0 mM; D-glucose, 5.5 mM; HEPES, 20 mM pH 7.4). Uptake was initiated by adding 50 μl of 100 μM L-[³H]arginine (NEN; 42.0 Ci/mmol, 1.0 mCi/ml) or 50 μl of 100 μM L-[³H]proline (NEN; 45.0 Ci/mmol, 1.0 mCi/ml) in prewarmed HEPES-buffered Krebs solution to each well. At specified time points, the label was removed and the macrophages were washed three times with ice-cold 10× phosphate-buffered saline (PBS) containing 10 mM unlabeled L-arginine or L-proline. Radioactivity in formic acid cell digests was measured by liquid scintillation counting. Initial experiments were performed with D-[¹⁴C]mannitol (NEN) to ensure that we were measuring uptake and not adherence of the label to the surface of the macrophages. Recovery of D-[¹⁴C]mannitol in the cell lysates was <0.01%. Macrophage protein was determined using the DC Assay (Bio-Rad). Results are expressed as annomoles of L-[³H]arginine or L-[³H]proline per milligram of total macrophage protein.

MCAT1 and MCAT2B RT-PCR. Total RNA from 3.0×10^6 resting, LPS–IFN-γ-stimulated, BCG-infected, or IFN-γ-stimulated–BCG-infected J774.1 macrophages was isolated using Trizol Reagent (Life Technologies) as specified by the manufacturer. cDNA was synthesized from the total RNA using oligo(dT) primers supplied in the SuperScript Preamplification System for First Strand cDNA Synthesis Kit (Life Technologies). Once the cDNA was isolated, quantitative reverse transcription-PCR (RT-PCR; *Taq* Polymerase supplied by Roche Pharmaceuticals) was performed. Primers to murine β-actin (Clontech) were used as a quantitation control. The sequences of the primers used to amplify MCAT1 from the oligo(dT) cDNA were 5′-CAACAATAGGACCAAAACA CCC and 5′-CGAAGATGCTCAAGACAGGAAG. The sequences of the primers used to amplify MCAT2B were 5′-TCGTAACACAAGCCAGGTTGG and 5′-TTTCCCAATGCCTCGTGTAATC. In order to quantitate the levels of mRNA from each of the four samples, initial PCRs were performed using each primer set to determine the linear range and equivalent cDNA concentrations for the three different PCR reactions. The linear range for the β-actin PCRs with all four cDNA samples were between 27 and 33 cycles. Upon quantitation of the β-actin PCRs, it was determined that 30 ng of resting cDNA, 20 ng of LPS–IFN-γ-treated cDNA, 20 ng of BCG-infected cDNA, or 20 ng of IFN-γ-stimulated–BCG-infected cDNA displayed the same degree of intensity at each cycle. These were the concentrations of cDNA used for all subsequent PCR reactions. The linear range for the MCAT1 PCR was between 32 and 40 cycles for all four samples, and the linear range for the MCAT2B PCR was between 27 and 33 cycles for all four samples. The final PCRs used for quantitation were performed at 30 cycles using the β-actin primers, 35 cycles using the MCAT1 primers, and 30 cycles using the MCAT2B primers. Fold differences in MCAT1 and MCAT2B expression were normalized to resting macrophage expression.

Nitrite determination. The production of nitrite by resting, LPS–IFN-γ-stimulated, BCG-infected, or IFN-γ-stimulated–BCG-infected J774.1 macrophages was measured using a fluorometric assay developed by Misko et al. (22). Under acidic conditions, 2,3-diaminonaphthalene (DAN; Molecular Probes) can react with nitrite to produce the fluorescent product, 1-(*H*)-naphthotriazole. To perform the assay, 10 μl of freshly prepared DAN (0.05 mg/ml in 0.62 M HCl) was added to a 100-μl sample (1.5×10^5 macrophages/well in a 96-well plate). The reaction was protected from light and incubated for 10 min at 20°C. The reaction was then terminated with 5.0 μl of 2.8 N NaOH and read on a fluorescent plate reader with excitation at 365 nm and emission at 450 nm with a gain setting at 100%. A standard curve was created with freshly made sodium nitrite (Sigma) standards dissolved in culture medium. Misko et al. (22) demonstrated that the phenol red present in DMEM interferes with the sensitivity of this assay. Therefore, the DMEM we used for the assay did not contain phenol red (Gibco-BRL).

Immunocytochemistry. One million J774.1 macrophages were seeded into 12-well plates, with each well containing 18-mm coverslips (VWR Scientific). The cells were then allowed to adhere for 2 h. Following macrophage stimulation (as described above), the media were aspirated from the macrophages, and the cells were fixed for 30 min at room temperature with freshly prepared 3.8% paraformaldehyde (Sigma) in PBS. The fixed macrophages were washed three times with PBS containing 5.0% sucrose and permeabilized with 0.2% Triton X-100 (Fischer Scientific) for 1 min. The macrophages were then washed twice with PBS containing 5.0% sucrose and blocked twice in PBS containing 5.0% sucrose and 2.0% goat serum (Sigma) for 5 min. Coverslips containing the macrophages were incubated with primary antibodies at a 1:1,000 dilution for 1 h in a humidified 37°C incubator. All of the primary antibodies used in this study were raised in rabbits. The antibodies against MCAT1 and MCAT2B were generously sup-

plied by James Cunningham from Harvard Medical School (20). The antibodies against iNOS were supplied by Transduction Laboratories. Following primary antibody exposure, the cells were washed three times with PBS containing 5.0% sucrose and 2.0% goat serum and then incubated with the secondary antibody (Texas red goat anti-rabbit immunoglobulin G [IgG] conjugate; Molecular Probes) at a dilution of 1:100 for 1 h in a humidified 37°C incubator. The cells were then washed three times with PBS containing 5.0% sucrose and mounted onto slides with Neo-Mount (VWR Scientific).

The slides were viewed with an Olympus BX60 microscope containing a rhodamine filter to detect fluorescence. To obtain fluorescent and phase-contrast images, an Optronics DEI-750 charge-coupled device camera with a SONY digital still recorder and digital interface board were used. Images were imported into and processed in Adobe Photoshop.

FACS analysis. A total of 1.0×10^6 J774.1 macrophages were plated into 12-well tissue culture plates and allowed to adhere for 2 h. Following macrophage stimulation (as described above), the cells were scraped from the bottom of the wells and centrifuged at 2,000 rpm in a microfuge for 10 min. The medium was then removed, and the cells were fixed for 30 min at room temperature with freshly prepared paraformaldehyde. The cells were exposed to antibodies and washed as described above. The secondary antibody used for fluorescence-activated cell sorting (FACS) analysis was a fluorescein-labeled goat anti-rabbit IgG conjugate (Molecular Probes). After the final wash following secondary antibody exposure, the cells were resuspended in 500 μl of PBS. The cells were then analyzed by FACS using a FACSCaliber (Becton Dickinson) equipped with a 488-nm argon ion laser and a 530-nm band-pass filter. The mean fluorescent intensity for each sample was acquired and analyzed with CellQuest software.

RESULTS

Uptake of L-arginine is enhanced in IFN-γ-stimulated J774.1 macrophages infected with BCG. To evaluate the effect of *M. bovis* BCG infection on the L-arginine-dependent NO response of murine macrophages, we sought to determine if BCG is able to stimulate L-arginine transport by J774.1 macrophages cotreated with IFN-γ. Previous studies have demonstrated that extracellular L-arginine is required for the production of NO by LPS–IFN-γ-activated murine macrophages (13). After 4 h of treatment, LPS–IFN-γ-treated macrophages accumulated twofold more L-[3H]arginine compared to resting (untreated) macrophages over a period of 10 min (Fig. 1A). No change in uptake (compared to resting macrophages) was detected upon treatment of the macrophages with IFN-γ, alone and a slight increase in uptake was detected upon treatment of the macrophages with LPS alone (Fig. 1A). These results are consistent with previously published results (2, 4). Infection with BCG did not stimulate L-[3H]arginine uptake, but a twofold increase in uptake was found upon simultaneous IFN-γ treatment and BCG infection of the macrophages (Fig. 1B). Therefore, BCG is able to replace LPS in IFN-γ-treated macrophages, leading to enhanced L-arginine uptake.

To gain insight into whether mycobacteria must be metabolically active to promote enhanced L-[3H]arginine uptake by IFN-γ-stimulated–BCG-infected macrophages, we treated the macrophages with heat-killed mycobacteria. Upon treatment of the macrophages with either heat-killed BCG or heat-killed *M. tuberculosis* plus IFN-γ for 4 h, a twofold stimulation of uptake was detected (Fig. 1C). Therefore, intact, metabolically inactive mycobacteria are able to stimulate L-arginine uptake by IFN-γ-cotreated macrophages. This suggests that a component(s) of the nonmetabolizing mycobacteria is responsible for L-arginine uptake stimulation by IFN-γ–LAM-treated macrophages. To ensure that the results we described here are specific for the uptake of L-arginine, we performed uptake assays using L-[3H]proline as the substrate. There was no difference

detected in the uptake of L-proline under all conditions tested (Fig. 1D).

Purified LAM from BCG or *M. tuberculosis* Erdman is unable to stimulate L-arginine transport by IFN-γ-primed macrophages. We have demonstrated that intact, metabolically inactive mycobacteria are able to stimulate L-arginine uptake by IFN-γ-stimulated macrophages (Fig. 1C). This suggests that a component of these nonmetabolizing mycobacteria must be responsible for enhanced L-arginine uptake. One such molecule is LAM. LAM is structurally similar to bacterial LPS and is secreted upon infection with *M. tuberculosis* (1). LAM is anchored in the plasma membrane of the mycobacterial cell envelope and traverses the mycobacterial cell wall (15).

To determine if LAM is the mycobacterial component responsible for the stimulation of L-arginine uptake by IFN-γ-costimulated J774.1 macrophages, the macrophages were treated for 4 h with 10 μg of either BCG or *M. tuberculosis* Erdman LAM and IFN-γ (kindly supplied by John Belisle) per ml. Figure 2 demonstrates that neither LAM molecule is able to stimulate uptake of L-arginine by IFN-γ-cotreated J774.1 macrophages. The uptake of L-arginine by both categories is the same as the uptake of L-arginine by resting macrophages (Fig. 1A). These results suggest that other preexisting mycobacterial components, such as the mycobacterial cell wall or mycobacterial antigens, are responsible for the enhanced uptake by IFN-γ-cotreated macrophages.

The expression of both MCAT1 and MCAT2B is altered upon activation and/or infection of J774.1 macrophages. Next, we sought to determine if L-arginine transport by resting, LPS–IFN-γ-stimulated, BCG-infected, or IFN-γ-stimulated–BCG-infected J774.1 macrophages is controlled at the level of transcription by using RT-PCR. MacLeod and coworkers (16) previously demonstrated via RT-PCR that there is a decrease in the expression of MCAT1 and an increase in expression of MCAT2B upon LPS–IFN-γ treatment of J774.1 macrophages. Our RT-PCR results are consistent with those data; the expression of MCAT1 by LPS–IFN-γ-activated macrophages is 4-fold lower than that of resting macrophages and the expression of MCAT2B is 1.7-fold higher than resting macrophages (Fig. 3, lane 2). These results are also consistent with the twofold increase in L-arginine uptake detected in LPS–IFN-γ-activated macrophages (Fig. 1A). Curiously, the expression of MCAT1 is elevated 3.8-fold and the expression of MCAT2B is elevated 2.4-fold in BCG infected macrophages (Fig. 3, lane 3). This was unexpected because the uptake of L-arginine by BCG-infected macrophages is the same as that of resting macrophages (Fig. 1B). Finally, the expression of MCAT1 by IFN-γ-stimulated–BCG-infected macrophages is the same as that of resting macrophages, and the expression of MCAT2B is twofold higher than that of resting macrophages (Fig. 3, lane 4). This is consistent with the uptake results, since the uptake of L-arginine by IFN-γ-stimulated–BCG-infected J774.1 macrophages is twofold higher than that of resting macrophages (Fig. 1B).

NO production by BCG-infected macrophages can be detected at 24 h postinfection and requires IFN-γ costimulation. Because the transport of extracellular L-arginine is enhanced in IFN-γ-stimulated macrophages infected with BCG, we sought to discover if the production of NO is also enhanced in these macrophages at 4 h postinfection as well. Chan et al. (7) dem-
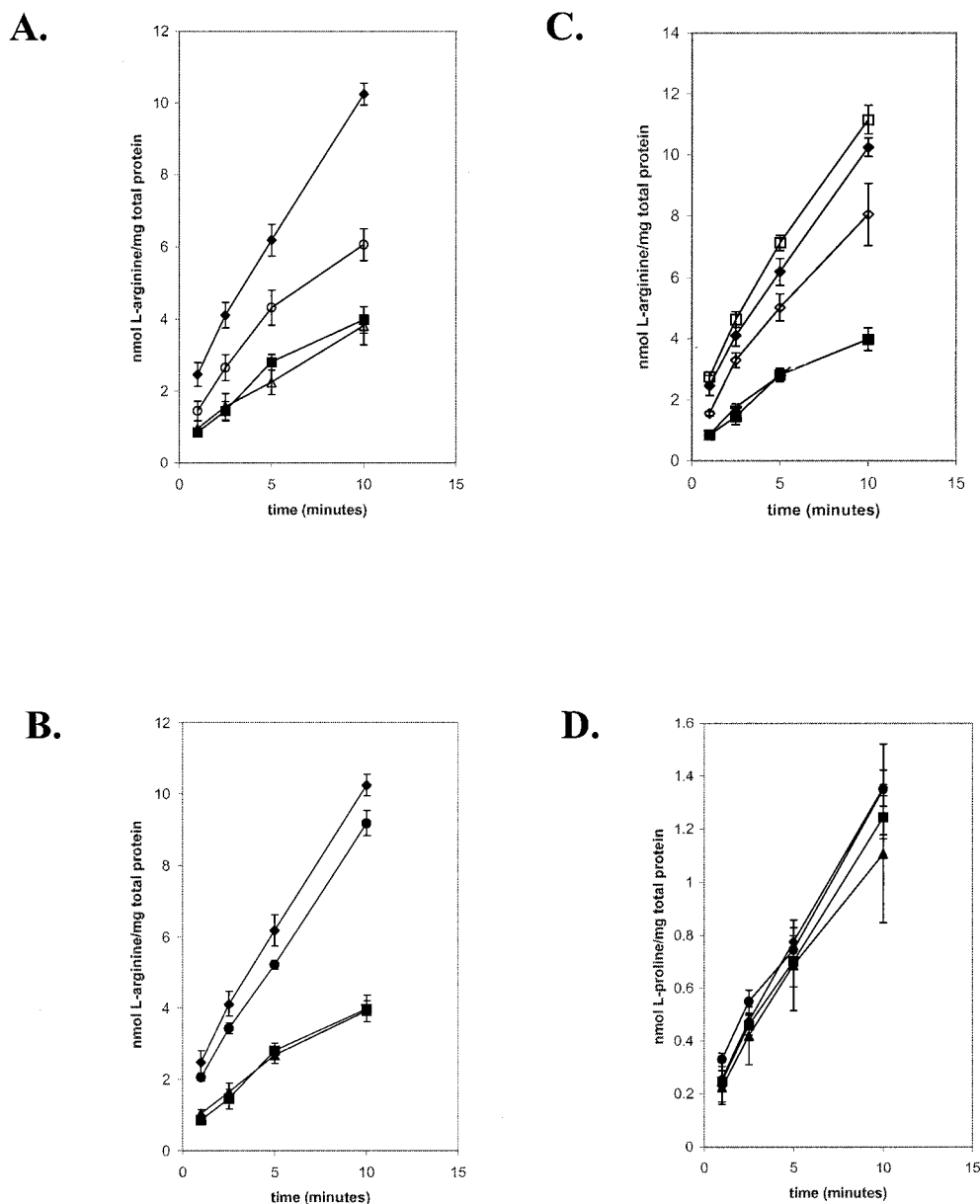
FIG. 1. Uptake of L-[³H]arginine (A, B, and C) and L-[³H]proline (D) by resting (■), LPS–IFN-γ-stimulated (◆), LPS-treated (○), IFN-γ-treated (△), BCG-infected (▲), BCG-infected–IFN-γ-treated (●), heat-killed BCG-infected–IFN-γ-treated (◇), and heat-killed *M. tuberculosis*–IFN-γ-treated (□) J774.1 macrophages. Uptake is expressed as nanomoles of substrate per milligram of total protein (see Materials and Methods). Two independent experiments with each amino acid were performed in duplicate for each strain.

onstrated that the generation of NO by activated macrophages is believed to be required to control mycobacterial infection in the murine system. To examine the production of NO by J774.1 macrophages, we chose to use a fluorometric assay as opposed to the more popular Griess assay. The fluorometric assay, developed by Misko and colleagues (22), is 50 to 100 times more sensitive than the Griess assay. For this assay, DAN reacts with nitrite to produce the fluorescent product 1-(*H*)-naphthotriazole which can be detected on a fluorescent plate reader.

Resting, LPS–IFN-γ-activated, BCG-infected, and IFN-γ-treated–BCG-infected macrophages were exposed to the DAN reagent, and the fluorescence was measured. The fold differences in nitrite production at 4 h are depicted in Fig. 4A. LPS–IFN-γ-activated macrophages produced 2.5 more nitrite than did resting macrophages. Macrophages infected with BCG, with or without IFN-γ costimulation, did not show significant increases in the production of nitrite by 4 h postinfection. The results from this assay demonstrated that BCG infection does not lead to the production of nitrite by J774.1 macrophages at 4 h postinfection. LPS is a more potent inducer of NO production 4 h after stimulation than BCG.

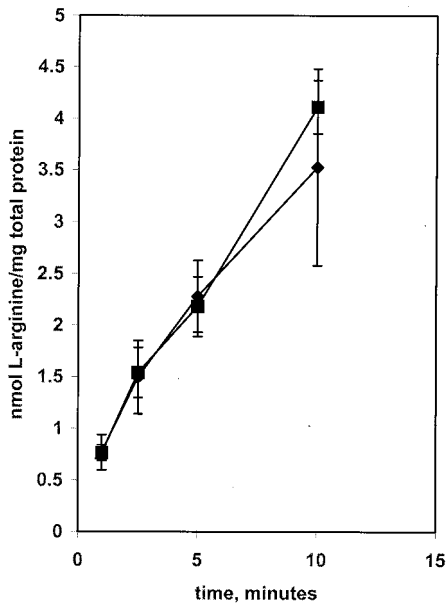Chan et al. (7) demonstrated that BCG infection of macrophages pretreated with IFN-γ for 12 to 16 h leads to the

FIG. 2. Uptake of L-[³H]arginine by IFN-γ-primed J774.1 murine macrophages treated with either BCG LAM (■) or *M. tuberculosis* Erdman LAM (◆) for 4 h.

production of NO at 24 h postinfection. To confirm that BCG was able to stimulate nitrite production by IFN-γ-costimulated macrophages, we examined the production of nitrite by resting, LPS–IFN-γ-activated, BCG-infected, or IFN-γ-treated–BCG-infected macrophages at 24 h postinfection. The fold differences in nitrite production after 24 h are depicted in Fig. 4B. LPS–IFN-γ-activated macrophages produced sixfold more nitrite than did resting macrophages. Nitrite production by
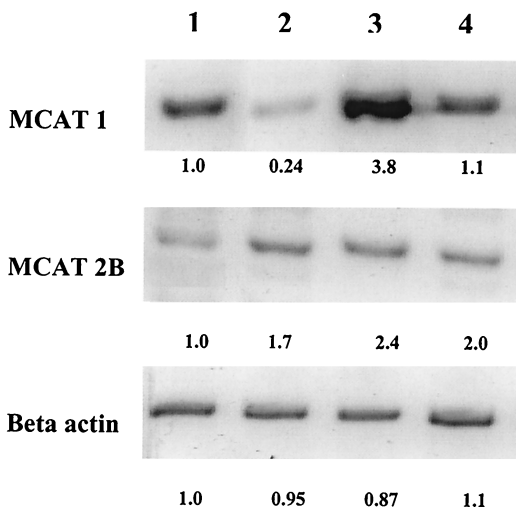

FIG. 3. RT-PCR of MCAT1 and MCAT2B from resting (lane 1), LPS–IFN-γ-stimulated (lane 2), BCG-infected (lane 3), or IFN-γ-stimulated–BCG-infected (lane 4) 1774.1 macrophages. β-Actin was used as a quantitative control (see Materials and Methods). Fold differences in expression of MCAT1, MCAT2B, and β-actin are listed below each sample and are normalized to resting macrophage expression levels.
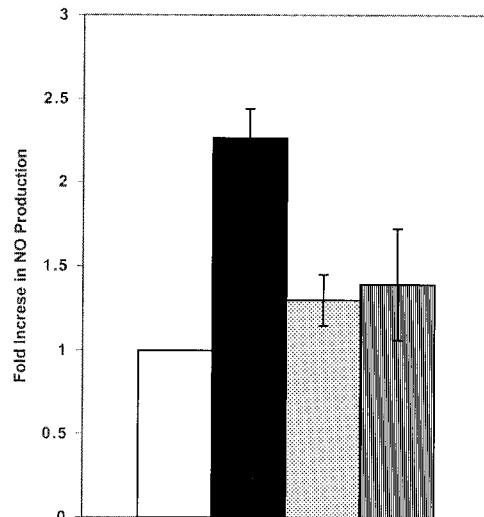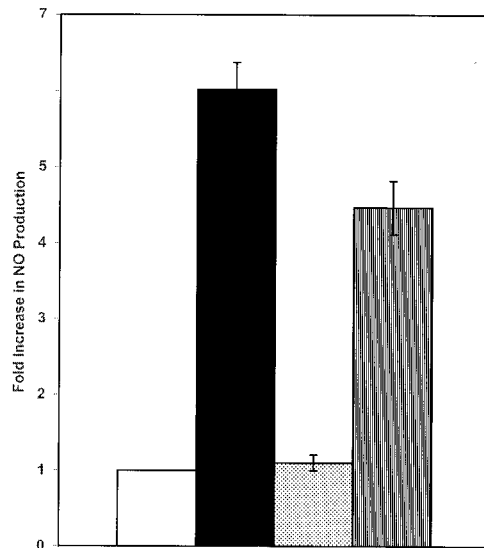

FIG. 4. Fold increase in the production of NO as measured by the DAN assay (see Materials and Methods) by resting (white bars), LPS–IFN-γ-stimulated (black bars), BCG-infected (dotted bars), and BCG-infected–IFN-γ-treated (vertically lined bars) J774.1 macrophages at 4 (A) or 24 (B) h poststimulation.

BCG-infected macrophages was the same as that of resting macrophages. In contrast to the results seen at 4 h, macrophages infected with BCG and costimulated with IFN-γ produced 4.5-fold more nitrite than did the resting macrophages. The results from this assay demonstrated that nitrite production by BCG-infected macrophages at 24 h requires IFN-γ costimulation.

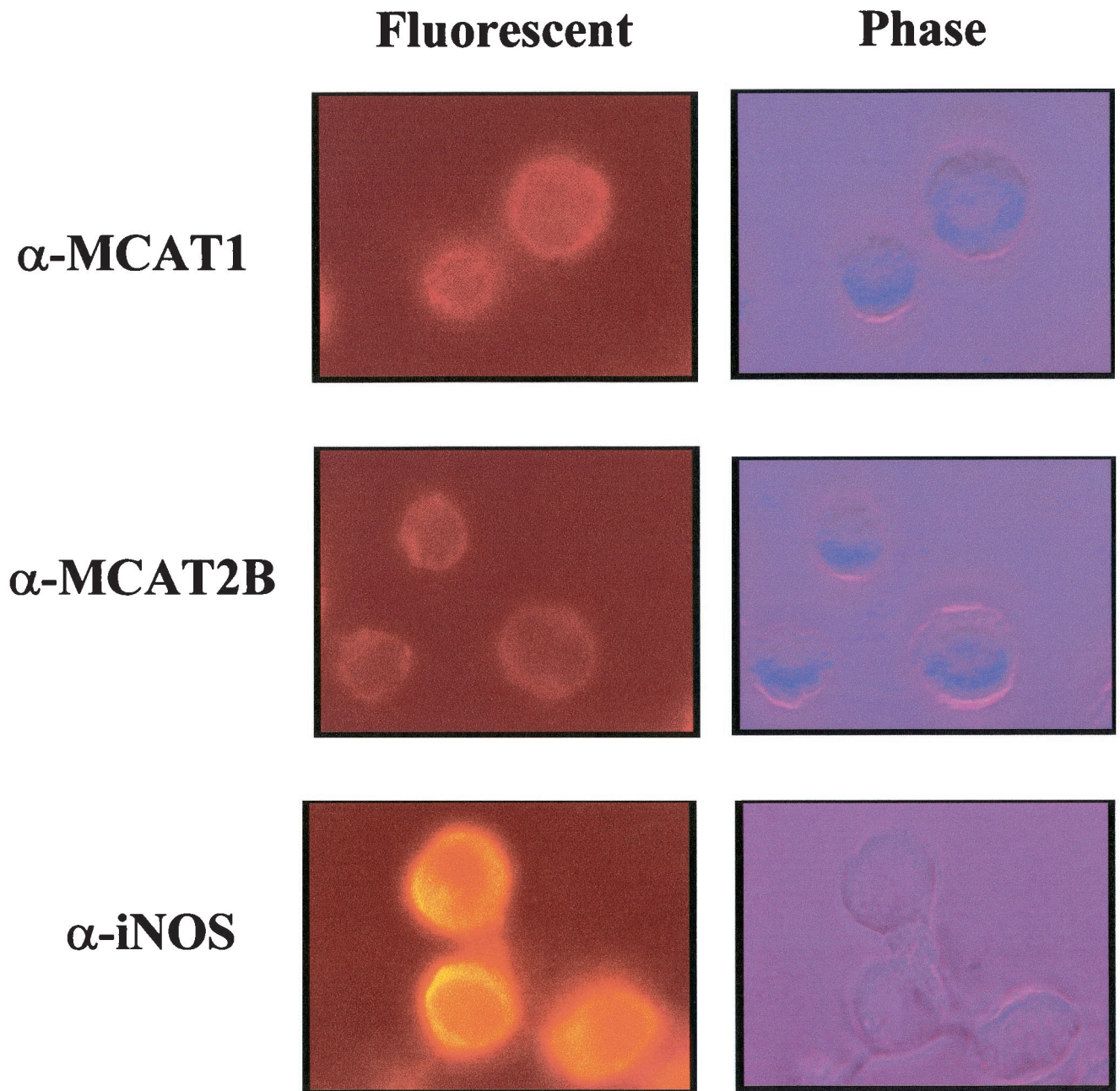**The level of MCAT2B protein expression is not sufficient for the increases in L-arginine uptake detected in LPS–IFN-γ-**

Fluorescent   Phase

FIG. 5. Representative immunocytochemical staining of LPS–IFN-γ-stimulated J774.1 macrophages with α-MCAT1, α-MCAT2B, and α-iNOS antibodies. Concurrent phase-contrast images (Phase) are also shown.

treated or BCG-infected–IFN-γ-treated J774.1 macrophages. In order to understand how the uptake of L-arginine and the production of NO by activated macrophages is regulated, we sought to quantify the MCAT1, MCAT2B, and iNOS proteins present in J774.1 macrophages under various conditions. Figure 5 depicts a representation of our immunocytochemical staining using LPS–IFN-γ-cotreated macrophages exposed to primary antibodies against MCAT1, MCAT2B, and iNOS. Figure 6 displays the results of the FACS analysis.

The protein expression of iNOS under the conditions tested mirrors the results obtained from the nitrite production stud-ies. Specifically, the protein expression of iNOS and the pro-duction of nitrite by LPS–IFN-γ-stimulated macrophages are both approximately 2.5-fold higher than that of resting macro-phages after 4 h of stimulation (Fig. 4 and 6). The iNOS expression and nitrite production by BCG-infected macro-phages (with or without IFN-γ) was lower than expected (1.3- to 1.5-fold greater than that of resting macrophages for both expression and nitrite production; Fig. 4 and 6). The data from the FACS analysis also demonstrated that there is no differ-ence in the expression of MCAT1 (the general L-arginine per-mease) under all conditions tested. Although there was an
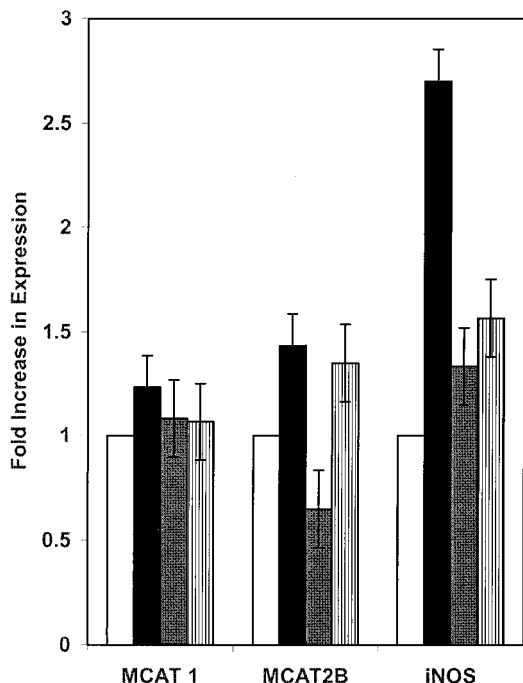
FIG. 6. Fold increase in expression of MCAT1, MCAT2B, and iNOS in resting (white bars), LPS–IFN-γ-stimulated (black bars), BCG-infected (dotted bars), and BCG-infected–IFN-γ-treated (vertically lined bars) J774.1 macrophages as determined by FACS analysis.

FIG. 7. Uptake of L-[³H]arginine by resting (■), LPS–IFN-γ-stimulated (◆), resting–cycloheximide-treated (▲), LPS–IFN-γ-stimulated and cycloheximide-treated (●) J774.1 macrophages. Uptake is expressed as nanomoles of substrate per milligram of total protein (see Materials and Methods). Two independent experiments with each amino acid were performed in duplicate for each strain.

increase in the expression of MCAT2B in both the LPS–IFN-γ-treated macrophages (1.4-fold over that of resting macrophages) and the BCG-infected–IFN-γ-treated macrophages (1.35-fold over that of resting macrophages), the increases in expression were not sufficient for the 2-fold increases in L-arginine uptake we detected.

Wileman et al. (32) have previously demonstrated that de novo protein synthesis is required for the uptake of L-arginine by LPS–IFN-γ-activated rat smooth muscle cells. To determine if the induction of L-arginine uptake by LPS–IFN-γ-activated macrophages is due to de novo protein synthesis, we treated the macrophages with cycloheximide after macrophage activation to block protein synthesis. After cycloheximide treatment, we performed uptake assays with L-[³H]arginine as the substrate. After 10 min, there was no difference in the uptake of L-[³H]arginine between cycloheximide-treated and untreated, resting macrophages (Fig. 7). This finding is consistent with the results of Wileman et al. Surprisingly, the enhanced uptake of L-[³H]arginine detected in LPS–IFN-γ-activated macrophages was not completely abolished to resting levels (Fig. 7). This suggests that the de novo protein synthesis of MCAT2B is not solely responsible for the enhanced uptake of L-arginine by activated macrophages.

DISCUSSION

The aim of our studies was to determine the impact of *M. bovis* BCG infection on the L-arginine-dependent NO pathway in J774.1 murine macrophages. To this end, we have demonstrated that BCG can replace LPS, leading to enhanced L-[³H]arginine uptake by IFN-γ-costimulated macrophages.

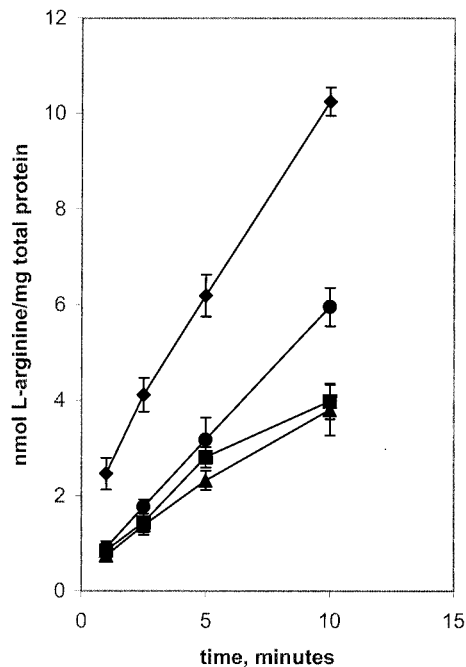We have also shown that intact, metabolically inactive mycobacteria are able to stimulate L-arginine uptake by IFN-γ-costimulated macrophages, whereas LAM was unable to do so. LAM is structurally similar to bacterial LPS and is secreted upon infection with *M. tuberculosis* (1). LAM is anchored in the plasma membrane of the mycobacterial cell envelope and traverses the mycobacterial cell wall (15).

Comparison of the LAM structures of *M. tuberculosis* H37Rv and Erdman (pathogenic strains of *M. tuberculosis*) to the LAM structures of *M. tuberculosis* H37Ra (a nonpathogenic strain of *M. tuberculosis*) and BCG demonstrated that all of the mannose residues in the molecule are capped, with the extent of capping being between 40 and 70% (18, 31). The LAM molecules are loosely classified into two groups: mannose-capped LAM and uncapped or arabinofuranosyl-terminated LAM. Generally, attenuated mycobacteria, such as *M. tuberculosis* H37Ra, produce arabinofuranosyl-terminated LAM, which is a potent inducer of NO production in murine macrophages (25). The virulent strains, *M. tuberculosis* H37Rv and Erdman, produce mannoyl-capped LAM (8). Although BCG is thought to be avirulent, it also produces LAM capped with several mannoyl residues (24). Thus, the results we detected with respect to the lack of L-arginine uptake stimulation by either LAM molecule were not surprising. It is possible that the effects seen with respect to enhanced L-arginine uptake by metabolically inactive mycobacteria may be due to LAMs associated with the mycobacterial cell walls or from other mycobacterial antigens because there was no enhanced uptake de-

tected in IFN-γ-costimulated macrophages exposed to BCG or *M. tuberculosis* Erdman LAM.

We have also shown that BCG infection of IFN-γ-costimulated macrophages does not lead to an induction of iNOS or an increase in NO production by 4 h postinfection. In contrast, IFN-γ–LPS-stimulated macrophages produce NO after 4 h of stimulation. A discrepancy arises when one considers that the production of NO has been shown to control mycobacterial infection by murine macrophages (7). These data also lead us to believe that there are different regulatory pathways involved in the induction of NO production by LPS versus mycobacteria. The regulatory pathway leading to the induction of NO by macrophages infected with mycobacteria is time delayed compared to macrophages stimulated with IFN-γ and LPS. In support of this, we have demonstrated that BCG-infected–IFN-γ-costimulated macrophages do produce enhanced NO at 24 h postinfection.

We also propose that because L-arginine transport was enhanced and no NO was produced by IFN-γ-stimulated macrophages infected with BCG at 4 h, L-arginine transport and the production of NO must be differentially regulated. Several studies have shown this to be the case. Shibazaki et al. (26) have demonstrated that L-arginine transport was detectable at doses of LPS that did not stimulate NO production. Bogle and colleagues demonstrated that J774.1 macrophages treated with LPS–IFN-γ displayed a greater induction of NO than did LPS-treated macrophages, whereas L-arginine uptake remained unchanged (4). The most compelling evidence of the differential regulation of L-arginine transport and NO production was from the lab of Wileman et al. (32). In their studies with J774.1 macrophages, they demonstrated that dexamethasone, a glucocorticoid, was able to selectively inhibit the production of NO by LPS–IFN-γ-activated macrophages but has no effect on enhanced L-arginine uptake.

It has been proposed that the enhanced L-arginine uptake seen in LPS–IFN-γ-activated macrophages is solely due to de novo protein synthesis of MCAT2B (3). Although we concur that de novo protein synthesis is required for enhanced uptake, our data suggest that there is another component involved. The results from the FACS analysis demonstrated that the levels of MCAT2B protein in both LPS–IFN-γ-stimulated and BCG-infected–IFN-γ-stimulated macrophages are not sufficient for the amount of L-arginine taken up under both of these conditions (Fig. 1 and 6). Also, the cycloheximide experiments show that the drug did not completely abolish L-arginine uptake to resting levels (Fig. 7). There are several ways in which this discrepancy can be explained. First, another uncharacterized transport protein could be present on the cell membrane, either derived from an unknown genetic locus or from splicing of a previously studied genetic locus. Second, there could be an alteration of the transport activity of one or more of the MCAT proteins in response to activation. Kakuda et al. (17) tested several putative L-arginine transporters for increased RNA and protein expression in response to LPS–IFN-γ stimulation, and none of them was responsive to activation except MCAT2B. Van Winkle et al. (30) performed kinetic experiments with MCAT1 and MCAT2B expressed in *Xenopus* oocytes and determined that both of these transporters are associated with more than one kinetically distinguishable transporter activity. These authors suggest that the transporter

proteins may exist in two kinetically different conformations or that transport accessory proteins may affect the kinetics of the transports. Therefore, the differences we detected between protein expression and L-arginine uptake in activated macrophages may be due to kinetic alterations of one or more of the MCAT transporters.

Another complexity of regulation of the MCAT proteins occurs at the level of MCAT gene expression. The levels of expression of MCAT1 and MCAT2B mRNAs in resting and LPS–IFN-γ-stimulated macrophages that we detected are consistent with previously published results (16). The mRNA expressions of MCAT1 and MCAT2B by BCG-infected macrophages are increased 3.8- and 2.4-fold over that of resting macrophages, respectively (Fig. 3). However, the protein levels of both MCAT1 and MCAT2B in BCG-infected macrophages is the same as that of resting macrophages, and the uptake of L-arginine by the infected macrophages is the same as that of resting macrophages (Fig. 1B and 6). This discrepancy may arise either from a decrease in the stability of MCAT1 and MCAT2B mRNAs or from an increase in the protein turnover of MCAT1 and MCAT2B in BCG-infected macrophages. Regardless of the mechanism, these results suggest that BCG infection of J774.1 macrophages leads to a perturbation of macrophage L-arginine uptake regulation, possibly through alternate signaling pathways. There are no inconsistencies between the protein expression, mRNA expression, and L-arginine uptake by BCG-infected–IFN-γ-stimulated macrophages (Fig. 1B, 3, and 5). Therefore, the IFN-γ-induced signaling must control the alterations in L-arginine uptake regulation created by BCG infection.

In closing, through these studies we have begun to understand the interaction between BCG and the macrophage with respect to the L-arginine-dependent NO pathway. These studies have also given us insight into the fine-tuning of L-arginine transport regulation and the regulatory interactions between the transport of L-arginine and the production of NO.

ACKNOWLEDGMENTS

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