Metabolism and Bioenergetics:

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Effect of Nicotine on Lung *S*-Adenosylmethionine and Development of *Pneumocystis* Pneumonia*

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Mehboob Shivji, Suzanna Burger, Camilo Andres Moncada, Allen B. Clarkson, Jr., and Salim Merali‡

From the Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York 10010

Because *S*-adenosylmethionine (AdoMet) is required by *Pneumocystis carinii in vitro*, *Pneumocystis* infection depletes plasma AdoMet of rats and humans, nicotine reduces AdoMet of guinea pig lungs, and smoking correlates with reduced episodes of *Pneumocystis* pneumonia (PCP) in AIDS patients, we tested the effect of nicotine treatment on PCP using a rat model. Intraperitoneal infusion of 400 μg of *R*-(+) nicotine kg^{-1} h^{-1} intraperitoneal for 21 days caused a 15-fold reduction in lung AdoMet although neither plasma nor liver were changed. Infusion of 4 and 400 μg kg^{-1} h^{-1} into immunosuppressed rats, beginning when rats were inoculated with *P. carinii*, caused 85 and 99.88% reductions, respectively, in *P. carinii* cysts at sacrifice 21 days later; *P. carinii* nuclei were reduced by 91.2 and >99.99%, respectively. This effect was reversed by concomitant administration of AdoMet with nicotine. Treatment with AdoMet alone increased infection intensity. We conclude that AdoMet is a critical and limiting nutrient for *Pneumocystis* thus can serve as a therapeutic target for PCP. Regarding the mechanism, nicotine treatment caused no change in rat lung activity of AdoMet synthesizing methionine ATP transferase activity nor was there any evidence of increased AdoMet utilization for methylation reactions. Except of a doubling of putrescine, nicotine treatment also did not change lung polyamine content. However, key polyamine anabolic and catabolic enzymes were upregulated, and there were corresponding changes in polyamine metabolic intermediates. We conclude that chronic nicotine treatment increases lung polyamine catabolic/anabolic cycling and/or excretion leading to increased AdoMet-consuming polyamine biosynthesis and depletion of lung AdoMet.

Pneumocystis is a fungal pathogen that infects the lungs of immunocompromised mammals causing a severe pneumonia known as PCP[1] (*Pneumocystis* pneumonia). Each species of mammal is associated with a particular species of *Pneumocystis*; for humans it is *P. jiroveci* and for rats, either *P. carinii* or *P. ratti* (1). Most human PCP cases are AIDS-related, and PCP is the most frequent opportunistic infection associated with AIDS (2, 3). However, all immunosuppressed persons are at risk including those treated with corticosteroids for rheumatic disease, cytotoxic agents for cancer, or immunosuppressive drugs for organ transplantation. PCP can also result from severe malnutrition. The rate of HIV infection associated with AIDS has declined in some developed countries because of specific prophylaxis and the wide use of effective anti-HIV drugs, but these medications are not routinely available for the vast majority of world populations where AIDS is rampant. PCP was thought to be infrequent in AIDS patients from developing countries, but recent data show that PCP occurs frequently in Africa (4–7). Recently the frequency of PCP cases, often associated with undiagnosed HIV, has increased (8). Even with access to good medical care, the mortality of PCP ranges from 10 to 40%, being higher in patients without AIDS. The two most effective drugs, pentamidine and the combination of trimethoprim and sulfamethoxazole (co-trimoxazole, TMP-SMZ), have significant side effects (9) and there is evidence of developing resistance (10–14). Clearly, there is a great need for new therapies that are more effective and less toxic (15).

AdoMet is a critical cellular metabolic intermediate. It plays a pivotal role as methyl donor in a myriad of biochemical processes including methyl group transfers for the formation of phosphatidyl choline, regeneration of methionine, methylation of phospholipids, and methylation of other small molecules (16). AdoMet-mediated methylation is an important regulatory mechanism for proteins, DNA and RNA (17). AdoMet also interacts with folate metabolism and thus all the reactions involving folate (18, 19). AdoMet is necessary for the synthesis of the essential polyamines spermidine and spermine (20, 21). AdoMet is synthesized in a one-step condensation of methionine and ATP catalyzed by AdoMet synthetase (methionine ATP transferase, MAT, EC 2.5.1.6). *Pneumocystis* is highly unusual in lacking this enzymatic activity (22). With the exception of *Pneumocystis*, a *Rickettsia* (23, 24) and an aberrant protozoan (25, 26), every other cell studied is able to synthesize AdoMet. *Pneumocystis* does require AdoMet and thus must obtain this key intermediate from its mammalian host. This requirement was first observed in culture and was supported by finding that infection with *Pneumocystis* causes ≥99% depletion of plasma AdoMet in an animal model of PCP (27) and in patients with PCP (28). Reduction of plasma AdoMet is such an unusual occurrence that measurement of AdoMet has potential as a minimally invasive, sensitive and specific diagnostic method for PCP and the rapid recovery of plasma AdoMet after initiation of effective treatment may serve as a

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‡ To whom correspondence should be addressed: Dept. of Medical and Molecular Parasitology, NY University School of Medicine, 341 East 25th St., New York, NY. Tel.: 212-263-6956; Fax: 212-263-8116; E-mail: merals01@popmail.med.nyu.edu.

[1] The abbreviations used are: PCP, *Pneumocystis* pneumonia; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CZE, capillary zone electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; IEF, isoelectric focusing; AdoMet, *S*-adenosylmethionine; i.p., intraperitoneal; ODC, ornithine decarboxylase; DC, decarboxylase; PMF, protein mass fingerprint.

measure of response to therapy (28).

Because of an asymmetric carbon atom at position 2 in the pyrrolidine ring, nicotine, 1-methyl-2-(3-pyridyl) pyrrolidine, exists as two optically active isomers. Nicotine produced by the tobacco plant is *S*(−) isomer, but, when burned in a cigarette, about 5% is pyrolytically converted to the *R*-(+) isomer (29). Regarding classical nicotine pharmacological effects, the *S*-(−) isomer is generally more potent (30). On average, about 90% of cigarette smoke is inhaled into the lung and, since the smoke from a cigarette contains about 6–8 mg of nicotine, and a cigar about 8-fold more, smokers can absorb considerable amounts of nicotine (31). In man, the major metabolite of nicotine is cotinine (70–80%) (32). Cotinine can be further metabolized by AdoMet-mediated methylation (33, 34) and *N*-methyl cotinine is a major urinary metabolite of nicotine (35). Studies with guinea pig tissue showed this methylation to be dependent on aromatic azaheterocycle *N*-methyltransferase (36) and to be specific for the *R*-(+) epimer. The nicotine K_m is 14.2 μM (37).

Nicotine has many pharmacological effects including modifying spontaneous nerve activity, heart rate, brain excitation, and blood pressure. Although high nicotine dosage can cause convulsions, anti-diuretic effects, and contraception, moderate dosage has been used to treat various illnesses ranging from cardiovascular problems to infections (31). The most effective nicotine application to date is treatment of veterinary parasitic helminth diseases: 19 mg of nicotine kg^{-1} per os treats *Fasciola*, *Taenia*, and *Ascaris* infections (38). The IC_{50} *in vitro* for various helminths ranges from 30 to 300 μM. Beyond veterinary application, nicotine and nicotine analogues were recently reported to be helpful for patients with Crohn's disease (39, 40), attention deficient/hyperactivity disorder, Alzheimer's disease, and schizophrenia (41–43). Efforts are being made to identify nicotine analogues with useful activity, less addictive potential, and less toxicity (44, 45).

Our interest in nicotine, AdoMet, and PCP began when we associated our findings that *Pneumocystis* requires AdoMet in culture and depletes plasma AdoMet in the rat model of PCP (22) with the results of a broad clinical study reporting a negative correlation between smoking and recurrent PCP (46) and older work showing a dramatic, lung-specific AdoMet reduction in guinea pigs treated with nicotine (29). From these associations, we developed the hypothesis that smoking protects against PCP by the action of nicotine causing a reduction in lung AdoMet. Here we report rat model data supporting this hypothesis and data suggesting the mechanism involved in the effect of nicotine on lung AdoMet.

EXPERIMENTAL PROCEDURES

Chemicals and Supplies—Ultra-pure AdoMet, α-cyano-4-hydroxycinnamic acid solution (CHCA), spermidine, spermine, putrescine, and *R*-(+) nicotine were from Sigma-Aldrich. AccQ.Fluor reagent kits were from Waters Corp. (Milford, MA). Urea, CHAPS, DTT, pharmalyte, glycerol, iodoacetamide, Immobiline Dry Strip (IPG), Plus One silver stain kits, and ExcelGel SDS XL 12–14 gels for two-dimensional separation were from Amersham Biosciences. Lyophilized trypsin powder was from Promega (Madison, WI). Pediatric suspension of trimethoprim and sulfamethoxazole (TMP/SMZ) was from Barre-National (Baltimore, MD). Dexamethasone sodium phosphate, 10 mg ml^{-1} for injection, was from Elkins-Sinn, Inc. (Cherry Hill, NJ). Cyanoacrylic surgical adhesive was from Henry Schein (Melville, NY)

Rat PCP Model—Specific pathogen-free S.D. rats (Taconic Farms, Germantown, NY) were housed in a barrier colony and maintained on multiple antibiotics to avoid other opportunistic infections, as previously described (48). The rats were also pretreated with a combination of trimethoprim and sulfamethoxazole (TMP/SMZ) for 21 days to reduce any latent *P. carinii* infection. This was done by adding 25 ml of a pediatric suspension of TMP/SMZ to each liter of drinking water yielding final concentrations of 0.2 mg of trimethoprim ml^{-1} and 1.0 mg of sulfamethoxazole ml^{-1}. After TMP/SMZ treatment, infusion pumps were implanted (see below) and delivered saline during the 7 days allowed for recovery before immunosuppression was begun by adding dexamethasone in the drinking water, 1.5 mg liter^{-1}. Inoculation with *P. carinii* was by intratracheal instillation of a lung homogenate from animals with PCP. Before use for inoculation, lungs were tested for other pathogens by Gram-stained impression smears and by streaking on blood agar plates to detect colony-forming fungi or bacteria (48). Inoculation was 4 days after initiation of immunosuppression and was repeated after an additional 2 days. The animals were sacrificed 21 days after the first inoculation. The degree of PCP was determined by counting of cysts and trophozoites in stained smears of lung homogenate as previously described (48).

Administration of Drugs by Infusion Pumps—Implantation and filling of pumps with 1.0-ml reservoirs and a nominal delivery rate of 0.15 ml day^{-1} (Veterinary Implant Products Division of Advanced Neuromodulation Systems, Plano, TX) were as previously detailed (48) and briefly described here. Steam-sterilized, saline-charged pumps were inserted into subcutaneous pockets in the dorsal thorax area that had been formed by a probe inserted from an incision in the skin of the back. The delivery capillary tubing was tunneled under the skin and inserted into the peritoneum through a small opening in the ventral midline just posterior to the sternum. The capillary was secured in place with cyanoacrylic surgical adhesive (VetBond). Incisions were closed with wound clips. The pump reservoir was accessed by inserting the needle of a 26-gauge infusion set through the skin into the fill port; the flexible tubing of infusion sets helps avoid movement of the needle thereby preventing damage to the fill port. Pump solutions were changed as follows. An infusion set needle was inserted into the fill port and any remaining solution was withdrawn with an attached syringe. The volume remaining in the pump allowed delivery rate calculation and the manufacturer-reported delivery rate of ~0.15 ml day^{-1} was confirmed. To add fresh solution, another infusion set was inserted and 3 ml of fresh solution was slowly injected into the pump flushing out any of the remaining old solution through the draining infusion set. The draining infusion set was then removed and 1.0 ml of fresh solution was injected into the pump as a refill.

The 1.0-ml volume of the pump reservoir allowed continuous delivery for >6 days without adding solution and, since analysis of the nicotine remaining in the pumps after 6 days indicated less than a 1% loss by degradation,[2] nicotine and saline solutions were replaced at 6-day intervals. Because AdoMet degrades rapidly, solutions containing AdoMet were replaced daily. At the time the pumps were implanted, the animals weighed 145–155 g. Immunosuppression by dexamethasone without *P. carinii* inoculation causes a typical weight drop to 120–130 grams after 4 weeks. *P. carinii*-inoculated animals typically weigh 90–100 g 21-days post-inoculation unless treated to suppress PCP. Nominal dose rates calculated at the beginning of the experiment are reported in "Results" although the dose rate can increase by as much as 20% by the end of the treatment period because of weight loss.

AdoMet Measurement—The AdoMet contents of lung, liver, and plasma samples were measured by HPLC analysis using Waters AccQ. Fluor derivatizing reagent as previously reported (27). For biological samples, the limit of detection is 0.5 nmol and linearity extends to 5,000 nmol. All samples were analyzed in triplicate, and the coefficient of variation ranged from 5 to 17%, depending on the amount of AdoMet in the sample.

Nicotine Measurement—Measurement of *R*-(+) nicotine in tissues and plasma was done using a modification of a published capillary zone electrophoresis (CZE) method (49). The apparatus was a P/ACE MDQ system equipped with a photodiode array detector allowing electropherograms to be monitored at 257 and 205 nm. We used an amine capillary kit (Beckman, Inc.; 50 mm inner diameter × 60 cm total length, 50 cm to the detector window). Sample preparation involved adding 20 μl of 10% perchloric acid to 80 μl of lung homogenate to precipitate the proteins that were removed by centrifugation at 5000 × *g* for 10 min. Supernatants were stored for up to 7 days at −20 °C before CZE analysis. Prior to analysis, samples were diluted 1:1 with water. The separation protocol was as follows: 2 min, 20 psi rinse using the kit "amine regenerator solution," 2 min, 20 psi rinse with Tris buffer (50 mM Tris, pH 8.0), sample injection and separation using 25 kV in reverse polarity mode for 7 min at 25 °C. Specificity was assured by demonstrating that *R*-(+) nicotine was resolved from all other peaks in cell extracts. Instrument precision was monitored by making triplicate injections from a single pooled standard. Linearity was demonstrated using a series of standard solutions over a range of 0.10–20.00 pmol.

Analytical Methods—Lung tissues (500 mg) from treated or normal lung were snap-frozen in liquid nitrogen and were crushed into a fine

[2] S. Merali, unpublished data.

powder using mortar and pestle. The powder was resuspended in 2 ml of buffer (150 mM KCl, 2 mM dithiothreitol, 25 mM HEPES, 5 mM $MgSO_4$,) and sonicated at 40 watts and 70% duty cycle for about 2 min, then centrifuged at 10,000 × *g* for 15 min. An aliquot of this lysate was used for Bio-Rad protein assay, and the protein from the rest of the lysate was used for the reaction mixture. Measurement of the AdoMet biosynthesis enzyme MAT was as previously described in (27). Presumptive SSAT activity (pmol of acetylspermidine (mg of protein)$^{-1}$ h^{-1}), AdoMet DC activity and ODC activity were as described previously (50–52). Spermidine, spermine, putrescine, and *N*-1-acetylated spermidine were analyzed by HPLC using Waters' AccQ.Fluor derivatizing reagent as previously reported (53).

Preparation of Samples for Proteomics Studies—The lungs of two nicotine-treated (400 μg of *R*-(+) nicotine kg^{-1} h^{-1}) and two saline-treated animals were washed aseptically three times with sterile saline then soaked for 15 min at 4 °C in saline. Proteins were extracted from 500 mg of aliquots of lung tissue as follows. After freezing with liquid nitrogen, tissue samples were ground to a powder using a mortar and pestle with liquid nitrogen in the mortar. The frozen powder was added directly to 0.8 ml of cold lysis buffer (9.8 M urea, 4% CHAPS, 10% glycerol, 15 mg ml^{-1} DTT, and 4% ampholytes) and sonicated at 40 watts and 70% duty cycle for about 2 min. The sonicate was centrifuged at 10,000 × *g* for 15 min to remove non-homogenized cartilage and other debris. A 10-μl aliquot of each lysate was used for a Bradford protein assay (Bio-Rad). Acetone with 10% trichloroacetic acid and 20 mM DTT was added to the balance to precipitate the proteins that were then collected by centrifugation at 10,000 × *g* for 30 min at 20 °C. The acetone/trichloroacetic acid/DTT solution was poured off and enough lysis buffer added to obtain a protein concentration of 3 mg ml^{-1}. The proteins were re-solubilized by sonication followed by shaking for 3 h at room temperature. After a final centrifugation at 10,000 × *g* for 15 min, the supernatant was collected for two-dimensional protein electrophoresis.

First Dimension Electrophoresis-Isoelectric Focusing (IEF)—IEF was performed using a MultiPhor II system Amersham Biosciences following the manufacturer's instructions. Immobiline dry strip gels (18 cm) were hydrated overnight (~16 h) in 350 μl of solution containing 2% CHAPS (w/v), 0.4% DTT (w/v), 7 M urea, 2 M thiourea, 2% carrier ampholytes solution (v/v), and 70 μg of sample protein. IEF was performed for 30 min at 150 V, 1 h at 300 V, 1.5 h at 1500 V, and 22.5 h at 3000 V to complete 70,000 VH. IEF focusing ranges were 4–7 and 4.5–5.5.

Second Dimension Electrophoresis (SDS-PAGE)—Prior to second dimension separation, Immobiline dry strip gel strips were soaked for 15 min in 10 ml equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 1% DTT, and 0.001% bromphenol blue in 0.05 M Tris-HCl buffer, pH 8.8) (54) and then soaked for 15 min in 10 ml of equilibration buffer containing 250 mg of iodoacetamide. Following the manufacturer's instructions, the Immobiline dry strip gel strips were placed on an Excel Gel SDS XL 12–14 (Amersham Biosciences) mounted in a Multiphor II electrophoresis unit connected to a Multi Temp III thermostatic circulator (Amersham Biosciences) set to 15 °C. Second dimension electrophoresis was run at 10 mA for 10 min, 20 mA for 20 min then at 40 mA for 1.5 h. The gels were silver-stained following the manufacturer's protocol.

Image Analysis of Two-dimensional Gels—Monochrome scans of gels were made at 200 dpi (HP model C7710A scanner) and stored in tiff format for input into the Z3 proteomics software package (Compugen, Jamesburg, NJ). The software automatically detected spots and assigned them numbers. Gel to gel variation was reduced by combining three gels per Immobiline dry strip gel range from each animal to produce Raw Master Gels (RMGs). RMGs of the two animals from the treatment group and the two from the control group were combined to produce Master Reference Gels, which were used to create differential displays. The software identified all differentially expressed protein spots in nicotine-treated animals relative to saline-treated controls including those up-regulated, down-regulated, newly expressed, or no longer expressed.

Predigestion Processing—Protein spots of interest were excised from the gel and destained by placing them in 1.5 ml of siliconized microcentrifuge tubes, washing with deionized water (500 μl, 6×), transferring into new 1.5-ml siliconized tubes, breaking into small pieces with a pipette tip (0.5–1 mm^2) and incubating in 20 μl of fresh destaining solution (equal parts 30 mM potassium ferricyanide and 100 mM sodium thiosulfate) until the brownish color disappeared (1–2 min). The destaining solution was decanted, and the gel pieces washed with water (20 μl, 5–6×) until all traces of yellow disappeared. They were then incubated for 20 min in 20 μl of 200 mM ammonium bicarbonate, washed with water (20 μl, 1×), and dehydrated with acetonitrile (30 μl, 2×) until opaque white. The gel pieces were dried in a vacuum centrifuge (30 min).

In-gel Trypsin Digestion—Purification of tryptic peptides followed a published method (55). A vial of 20 μg (833 pmol) of lyophilized trypsin powder was dissolved in 100 μl of 50 mM acetic acid and diluted to 16 ng μl^{-1} with 230 μl of 50 mM ammonium bicarbonate. A 20–30-μl volume of trypsin solution (depending on the amount of dried gel in the sample) was added to each set of dried gel pieces. Digestion was at 37 °C for 18–20 h, then the digests were cooled, held at 4 °C for 30 min, gently centrifuged for 10 s, sonicated in a 30 °C water bath for 5–6 min, and centrifuged at 12,000 × *g* for 2 min. The supernatants containing the tryptic fragments were transferred into 0.5-ml siliconized tubes.

MALDI-TOF MS—MALDI plates pre-spotted with α-cyano-4-hydroxycinnamic acid solution as a matrix were prepared as previously described (55). Protein Mass Fingerprint (PMF) data for each protein digest were obtained by MALDI-TOF MS using a Voyager Elite Applied Biosystems instrument with the following parameters: delayed extraction (DE); reflective (R); positive ion mode; accelerating voltage 30 kV; grid voltage 57%; mirror voltage ratio 1.08; guide wire 0 is 0.07%; extraction delay time 150 ns; acquisition range *m/z* 450–3500; and low mass gate 400 Da. The laser wavelength was 337 nm and repetition was at 1.5 Hz. The instrument produces a protonated ion, [M+M+]+. Final mass spectra were produced by averaging the data from 50–200 laser shots and processing the results with Data Explorer software to obtain accurate mono-isotopic peaks. Mass spectra were internally calibrated using these two fragments reliably produced by trypsin autodigestion: amino acid sequence 108–115 ([M+H]+ = 842.509 Da) and sequence 58–77 ([M+H]+ = 2211.104 Da). Blank gel experiments provided data for correction of spectral components derived from common contaminants (typically keratins).

Data Base Analysis—SWISS-PROT and NCBlnr data bases were searched for matches to corrected PMF using MS-FIT software (prospector.ucsf.edu/).

RESULTS

Delivery of Nicotine to Rat Lungs via Intraperitoneal Infusion—Since systemic nicotine had been reported to reduce lung AdoMet of guinea pigs (29), and systemic administration seemed more relevant for possible clinical application, we chose peritoneal delivery rather than delivering nicotine directly to the lungs. Because we use a rat model of PCP, we first tested whether nicotine infused into the peritoneum of rats reaches their lungs. Pumps were implanted in the suprascapular region of two groups of four animals each, and the delivery cannulas were positioned to deposit nicotine in the peritoneum. The pumps delivered saline for a 7-day surgical recovery period then chamber fluid was exchanged for either 10.5 or 0.105 mg of *R*-(+) nicotine tartrate ml^{-1} in saline which, with a delivery rate of 0.15 ml day^{-1}, resulted in nominal doses of 400 or 4 μg of *R*-(+) nicotine kg^{-1} h^{-1}, respectively. Since the reservoir was 1.0 ml, the pumps were refilled every 6 days with fresh solution. After 21 days of infusion, the animals were sacrificed, and the lungs analyzed for nicotine using a novel CZE method we developed. This method provides a linear relationship (r^2 = 0.9997) between the amount of standard and peak area over a range 0.10–20.00 pmol. A typical electropherogram from plasma of an animal treated with 400 μg of nicotine kg^{-1} h^{-1} is shown in Fig. 1. Peritoneal infusion for 21 days resulted in lung *R*-(+) nicotine concentrations of 62 ± 12.5 (S.D.) ng g^{-1} and 1.8 ± 0.5 ng g^{-1}, respectively, for the two dose rates.

Lung AdoMet Response to Intraperitoneal Infusion of Nicotine—A nominal dose of 400 μg of *R*-(+) nicotine kg^{-1} h^{-1} was infused into the peritoneum of a group of 15 animals. At 0, 1, 7, 14, and 21 days of treatment, we analyzed the lung AdoMet content of three rats randomly assigned for sacrifice and there was a progressive reduction compared with untreated controls (Fig. 2). Based on these results, a 21-day treatment period was chosen for the rest of the experiments. The control value of 8.2 ± 2.1 μg of AdoMet g^{-1} lung is similar to a previously reported value of 11.1 μg ml^{-1} in rats (56). The 11-fold decrease at 21 days is similar to the 9-fold decrease reported with guinea pigs treated with *R*-(+) nicotine but was achieved with half the nicotine dose rate (29).

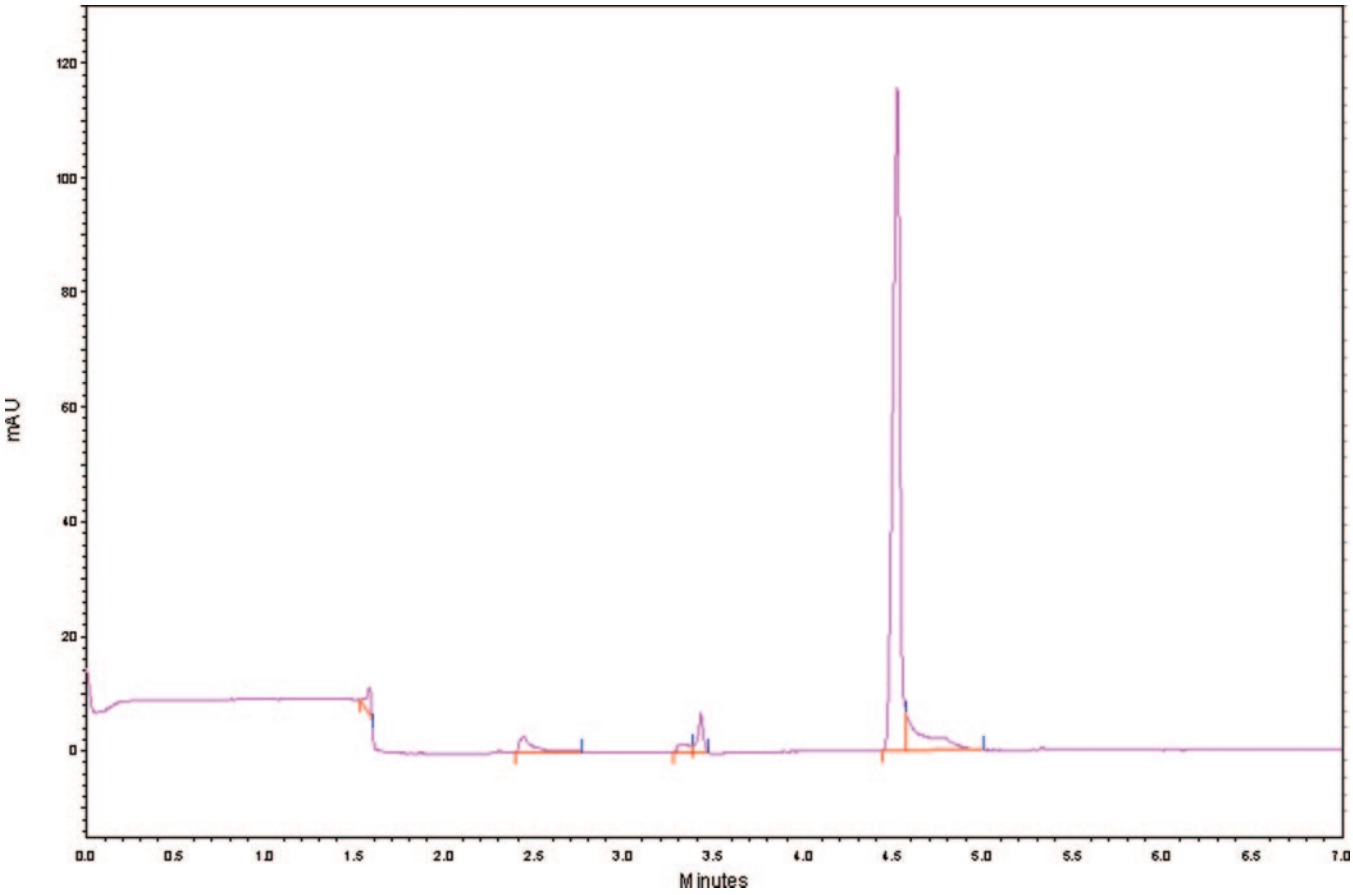

FIG. 1. **Electropherogram of *R*-(+) nicotine in plasma of an animal treated with 400 μg of nicotine kg^{-1} h^{-1} for 21 days.**

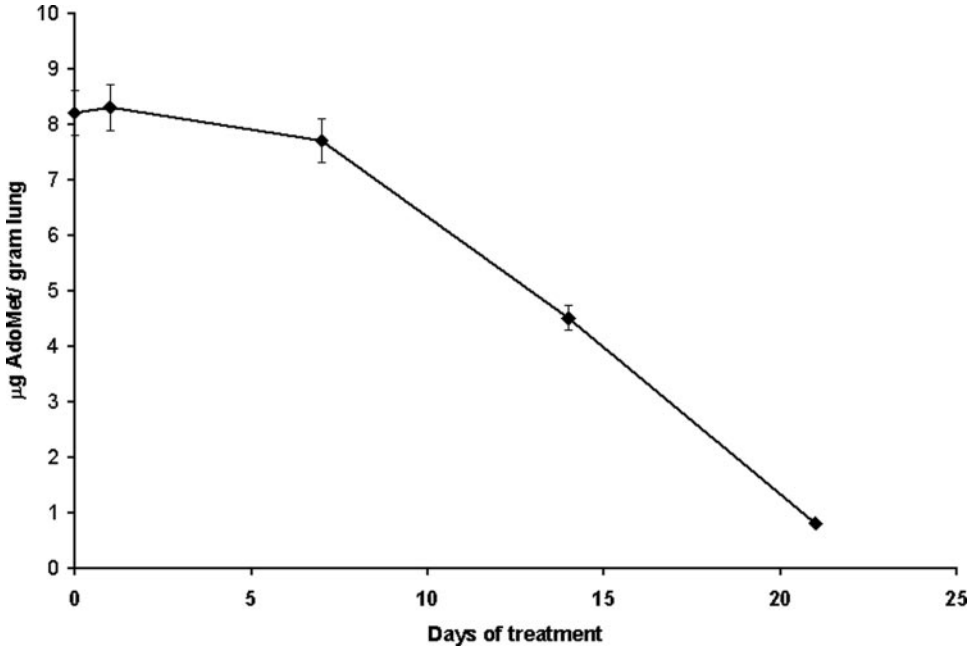

FIG. 2. **Time course of lung AdoMet depletion in response to nicotine.** Implanted pumps delivered a dose of 400 μg of *R*-(+) nicotine kg^{-1} h^{-1} to a group of 15 otherwise untreated animals. At times 0, 1, 7, 14, and 21 days of treatment, randomly chosen rats were sacrificed, and the lungs were removed for AdoMet analysis. Data points are means (± S.D.) from three independent experiments.

Lung, Plasma, and Liver AdoMet of Immunosuppressed Rats after Nicotine Infusion—Since our rat PCP model involves immunosuppression by dexamethasone, we measured the effect of nicotine on lung AdoMet of rats treated with dexamethasone. We also tested the specificity of this effect relative to plasma and liver. Pumps were implanted as above, and after the 7 day recovery period immunosuppression was initiated by adding dexamethasone to the drinking water (1.5 mg liter^{-1}). After an additional 7 days, the contents of the pumps in one group of 4 rats were changed to deliver 400 μg of nicotine kg^{-1} h^{-1}. The

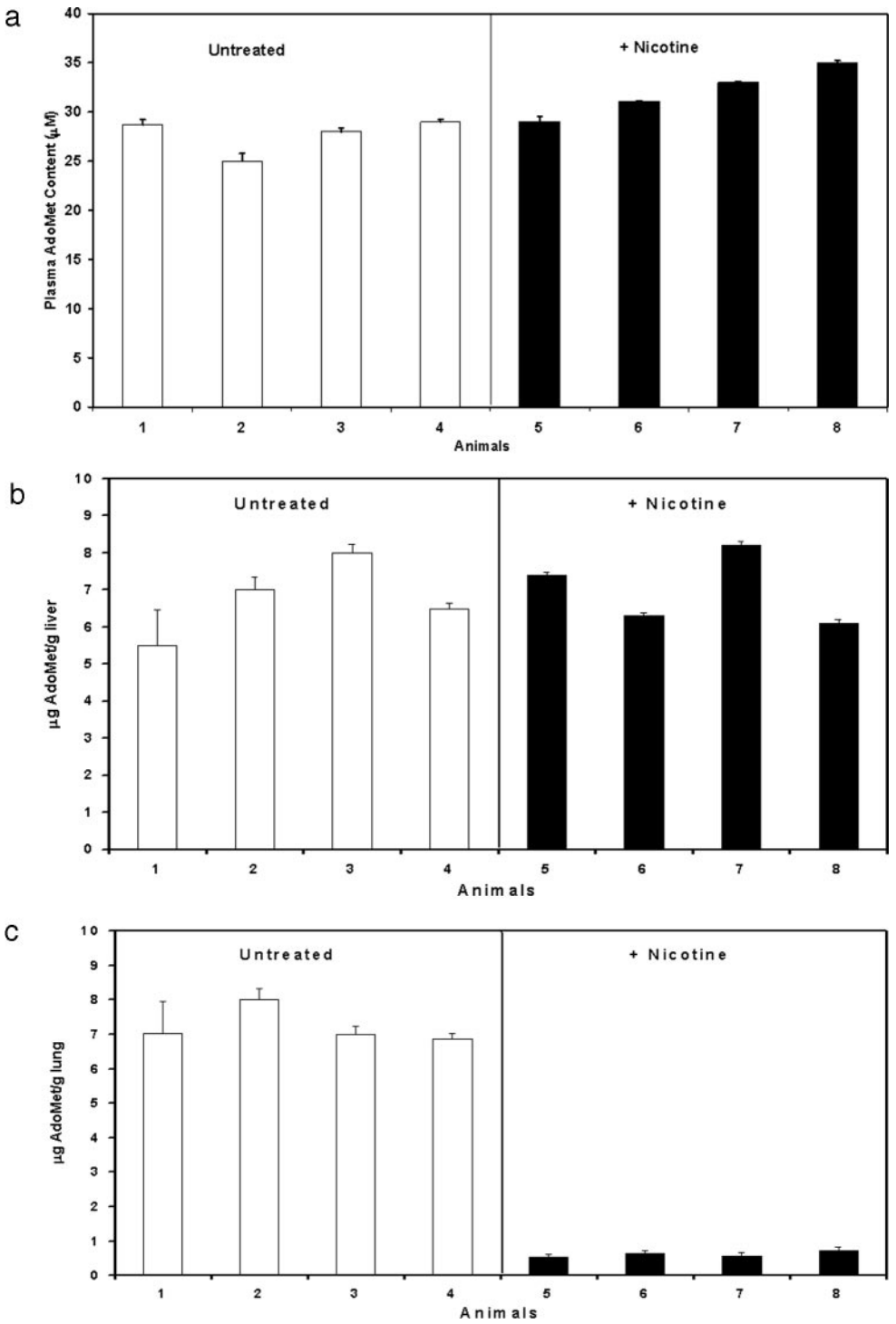

FIG. 3. **Intraperitoneal infusion of *R*-(+) nicotine and AdoMet content of plasma, liver, and lung.** Implanted infusion pumps were used to administer a nominal dose of 400 μg of *R*-(+) nicotine kg^{-1} h^{-1} or saline to two groups of four immunosuppressed but uninfected rats. After 21 days of treatment the AdoMet in plasma or tissue homogenate was analyzed. *Bar* represents the mean of three analyses of individual animals ± S.D. *a*, liver; *b*, plasma; *c*, lung.

other group continued with saline as controls. After 21 days of treatment, the animals were sacrificed and tissues collected for analysis. Fig. 3, *a* and *b* show that plasma and liver AdoMet were not significantly different between treated and control groups. However, lung AdoMet was reduced by 15-fold (Fig. 3*c*), an effect somewhat greater than observed without dexamethasone treatment (Fig. 2).

Nicotine Treatment of an Animal Model of PCP—We repeated the protocol with dexamethasone as above but inoculated animals with *P. carinii* at the same time the *R*-(+) nicotine infusion began. Groups of 5 rats were administered 4 or 400 μg of nicotine kg^{-1} h^{-1} and controls were infused with saline. The animals were sacrificed 21 days post-inoculation and the degree of PCP evaluated by counting *P. carinii* cysts and nuclei (nuclei counts include both intracystic bodies within cysts and trophozoites) (Table I). Compared with the saline-treated controls, animals administered 4 and 400 μg of *R*-(+) nicotine kg^{-1} h^{-1} intraperitoneal had reductions in cyst count of 84.92 and 99.88%, respectively, and nuclei count reductions of 91.18 and >99.99%, respectively.

TABLE I
Effect of R-(+) nicotine on development of PCP

Treatment was begun at the time the animals were inoculated with *P. carinii*. Animals were sacrificed 21-days post-inoculation and lung homogenates analyzed for *P. carinii* cysts and nuclei.

21-Day i.p. infusion dosage[a]	Mean cysts per lung $\times 10^6$ (±S.E.)	Mean nuclei per lung $\times 10^9$ (±S.E.)
Saline	6.3 (±2.05)	8.5 (±2.95)
4 μg of *R*-(+) Nicotine kg^{-1} h^{-1}	0.95 (±0.34)	0.75 (±0.18)
400 μg of *R*-(+) Nicotine kg^{-1} h^{-1}	0.0074 (±0.0009)	0.0014 (±0.0022)
400 μg of *R*-(+) Nicotine plus 1000 μg of AdoMet kg^{-1} h^{-1}	7.2 (±4.05)	11.8 (±7.4)

[a] $n = 3$.

TABLE II
Effect of AdoMet administration on development of PCP in the rat model

Treatment was begun at the time the animals were inoculated with *P. carinii*. Animals were sacrificed 21-days post-inoculation and lung homogenates analyzed for *P. carinii* cysts and nuclei.

21-Day i.p. infusion dosage[a]	Plasma AdoMet	Lung AdoMet	Mean cysts per lung $\times 10^6$ (±S.E.)	Mean nuclei per lung $\times 10^9$ (±S.E.)
	$\mu M \pm$ S.D.	$\mu M \pm$ S.D.		
Saline	28.6 (±1.6)	7.2 (±0.6)	5.5 (±0.8)	2.1 (±0.55)
1000 μg of AdoMet kg^{-1} h^{-1}	111.5 (±12.4)	15.4 (±2.6)	10.4 (±0.7)	3.8 (±0.87)

[a] $n = 3$.

Reversal of Nicotine Effect on PCP by Exogenous AdoMet—We tested our hypothesis that nicotine acts against *Pneumocystis* via deprivation of AdoMet by attempting to reverse the effect with exogenous AdoMet. An additional group of 5 animals was included in the experiment just above and AdoMet was co-administered with nicotine by including it in the pump solution to deliver 400 μg of *R*-(+) nicotine and 1000 μg AdoMet kg^{-1} h^{-1}. The cyst count for this group was 114% of the saline-treated control group and the trophozoite count 138% of the control group (Table I). Thus AdoMet did reverse the effect of nicotine supporting our hypothesis.

General Effect of Exogenous AdoMet on PCP—Because 1000 μg of AdoMet kg^{-1} h^{-1} seemed to more than reverse the effect of 400 μg of *R*-(+) nicotine kg^{-1} h^{-1}, we considered whether AdoMet could be a limiting factor in the growth of *Pneumocystis*, even in the absence of nicotine. We tested this by infusing *P. carinii*-inoculated rats with 1000 μg of AdoMet kg^{-1} h^{-1} beginning at the time of inoculation. Controls were infused with saline. At sacrifice 21 days post-inoculation, plasma AdoMet was ~4-fold greater than saline-treated controls and lung AdoMet ~2-fold greater (Table II). *P. carinii* cyst count and nuclei counts were both increased by ~2-fold (Table II). These data suggest that AdoMet is limiting for *P. carinii* growth *in vivo* and that the exogenous AdoMet we co-administered with nicotine likely overcompensated for the effect of nicotine. We do not know if a greater AdoMet dose would enhance *P. carinii* growth further.

Mechanism of AdoMet Depletion by Nicotine Treatment—The chronic nature of the effect of nicotine on lung AdoMet suggests that a cascade of processes may be involved. We have not explored initial events but have considered the mechanisms ultimately involved in the decline in lung AdoMet after 3 weeks of nicotine treatment. AdoMet is produced from methionine and ATP by methionine ATP transferase (MAT, AdoMet synthetase) and the observed reduction in AdoMet could have been caused by down-regulation of MAT. However, we found the mean MAT activity of the lungs from 3 untreated rats to be 3.1 ± 0.5 pmol mg $protein^{-1}$ min^{-1} and from 3 rats treated with 400 μg of *R*-(+) nicotine kg^{-1} h^{-1} intraperitoneal for 21 days to be very similar, 2.9 ± 0.7 pmol mg $protein^{-1}$ min^{-1}. Thus a change in MAT activity seems not to account for the decline in lung AdoMet. Because the major use of AdoMet is for various methylation reactions, we considered the possibility that an increase in methylation leads to lowered AdoMet. The methylation index of a tissue, ratio of AdoHcy (methylation endproduct) to AdoMet (methyl donor), is used as measure of changes in the rate of AdoMet driven methylation; an increase of the methylation index is taken to reflect increased AdoMet consumed by various methylating enzymes. The mean methylation indices of the lungs of three saline-treated controls and three rats treated with 400 μg of *R*(+)-nicotine kg^{-1} h^{-1} intraperitoneal were 19 ± 2 and 16 ± 2, respectively. This slight decrease with nicotine treatment indicates no change or a small reduction in the rate of AdoMet consumption by methylation pathways. We then applied the powerful global approach of proteomic analysis. Protein extracts were prepared from immunosuppressed control rats treated with saline for 21 days and immunosuppressed rats treated with 400 μg mg *R*-(+) nicotine kg^{-1} h^{-1} for the same period. Protein extracts were separated by two-dimensional gel electrophoresis, initially using the wide isoelectric focusing range of pH 4–7 for one dimension and SDS-PAGE for the other. These gels produced 3874 individual protein spots (example: Fig. 4, *top panel*). To gain resolution, we also ran gels using the narrow isoelectric focusing range of pH 4.5–5.5 (example: Fig. 4, *bottom panel*). Scans of silver stained were imported into the Z3 Proteomics Analysis Program, and raw master gels were produced by combining the scan results of three replicate gels for each isoelectric focusing pH range for each of the two lung protein extracts. Master gels for each pH range for each treatment group were produced by combining the raw master gels from the individual treatment and control animals (Fig. 5, pH 4.5–5.5 Master gels). These master gels were used to create differential display profiles (Fig. 6). Spots circled with *yellow* are proteins that were down-regulated by nicotine treatment; *blue*, up-regulated; *green circled with red*, newly expressed; and *magenta circled with red*, no longer expressed.

Out of the total of 21 differentially expressed spots, so far we have analyzed 12 by peptide mass fingerprinting (PMF) using data obtained from MALDI-TOF. The PMF data sets were used to search SWISS-PROT and NCBInr databases using MS-FIT software (prospector.ucsf.edu). Data from two spots produced direct hits by matching only single proteins in the databases: rat ornithine decarboxylase (ODC) and rat ubiquitin-conjugating enzyme. These spots are marked in Fig. 6. ODC is a critical polyamine biosynthesis enzyme and had previously been shown to be elevated in lung of rats treated with nicotine (57). Ubiquitin-conjugating enzyme is involved in protein turnover

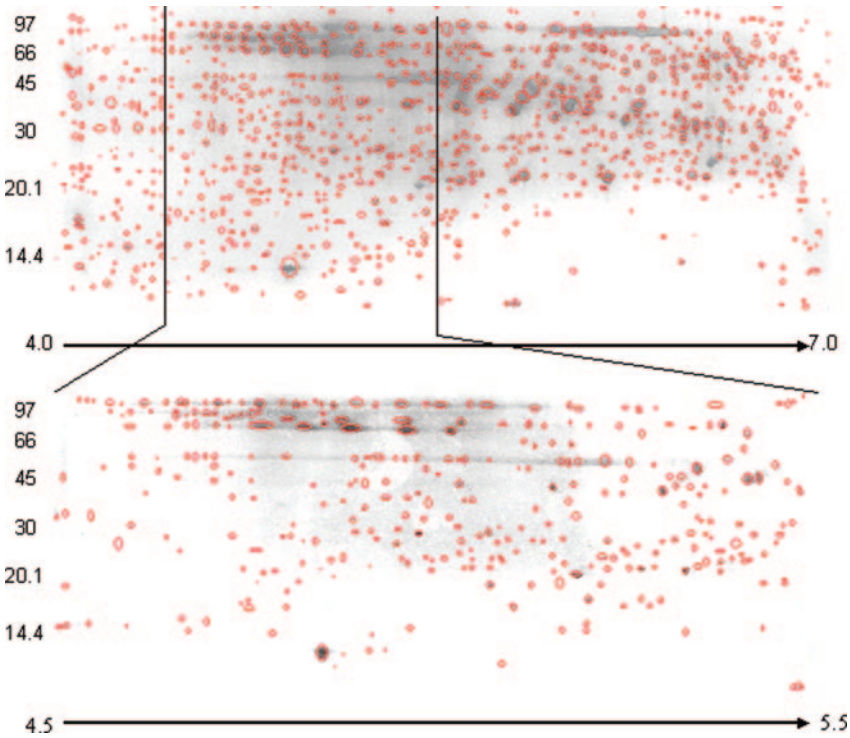

FIG. 4. **Lung proteome response to nicotine administration.** These two-dimensional silver-stained gels were prepared using proteins extracted from a lung of a rat-treated with 400 μg of *R*-(+) nicotine kg^{-1} h^{-1}. The *upper panel* is a global map (pH 4–7), and the *lower panel* is an ultrazoom map (pH 4.5–5.5). Proteins from the lung lysate were extracted by trichloroacetic acid precipitation, separated in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE. Analysis by Z3 two-dimensional software identified 1,895 proteins in the global map and 428 proteins in the ultrazoom map.

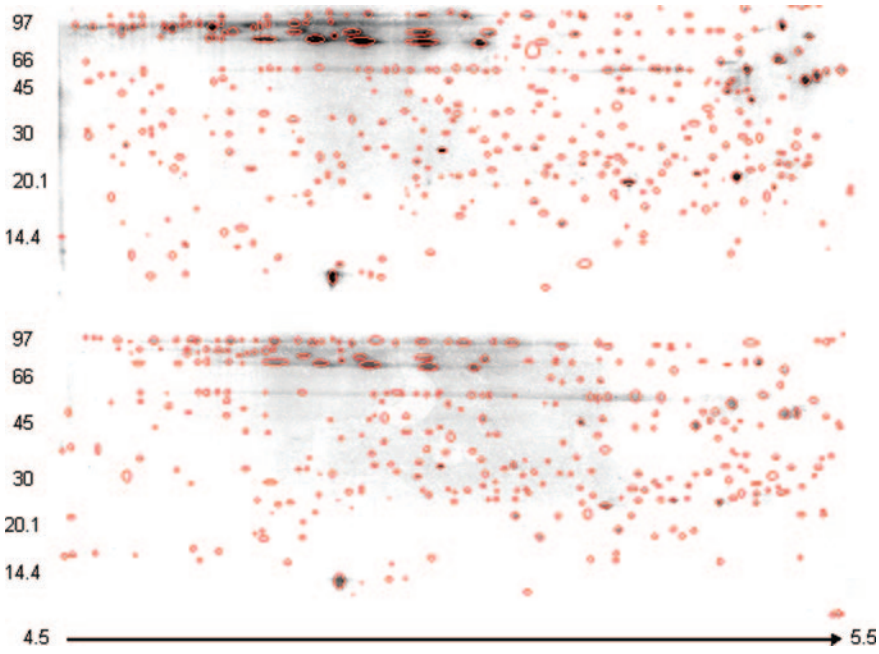

FIG. 5. **Master gels of control (*top*) and *R*-(+) nicotine-treated (*bottom*) rat lungs.** To correct for minor inconsistencies between gels, triplicate two-dimensional gels were prepared from each lung homogenate of the two nicotine-treated animals and from each homogenate of the two saline-treated controls. Images of the triplicate gels were combined by Z3 software to produce raw master gels for each animal. Master gels were created by combining the raw master gels from two nicotine-treated animals and similarly for master gels from the controls.

and could be expected to increase when protein expression changes; the PC12 cell line has been reported to up-regulate ubiquitin conjugating enzyme in response to nicotine exposure (58). Regarding those spots that appear to be differentially expressed according to nicotine treatment but produced trypsin fragment masses consistent with more than one protein in the databases, we are currently performing peptide sequence analysis using capillary column liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS) to obtain partial sequence information which, when combined with our PMF data, is expected to allow identification of many more spots.

Effects of R-(+) Nicotine on Polyamine Biosynthetic Enzyme Activities—ODC is a major regulatory enzyme for the polyamine biosynthesis pathway, a pathway that consumes AdoMet. The product of ODC, putrescine, is converted to the polyamines spermidine and spermine in sequential steps each using a molecule of AdoMet that has been decarboxylated by AdoMet decarboxylase. Thus if polyamine biosynthesis is increased, as suggested by the 14-fold increase in ODC protein detected by densitometry of the two-dimensional gels, AdoMet consumption will also be increased. However, ODC is also controlled by post-translation mechanisms so an increase in ODC protein does not necessarily mean an increase in ODC activity. Therefore we assayed ODC enzymatic activity in homogenates of lungs from three rats infused with 400 μg of *R*-(+) nicotine kg^{-1} h^{-1} for 21 days and three controls infused with saline; all six rats were immunosuppressed but none were inoculated with *P. carinii*. Using our previously described assay (50), we found activities of 78 ± 14 nmol putrescine g^{-1} h^{-1} in nicotine-treated lungs and 4.1 ± 1.1 in saline controls, a

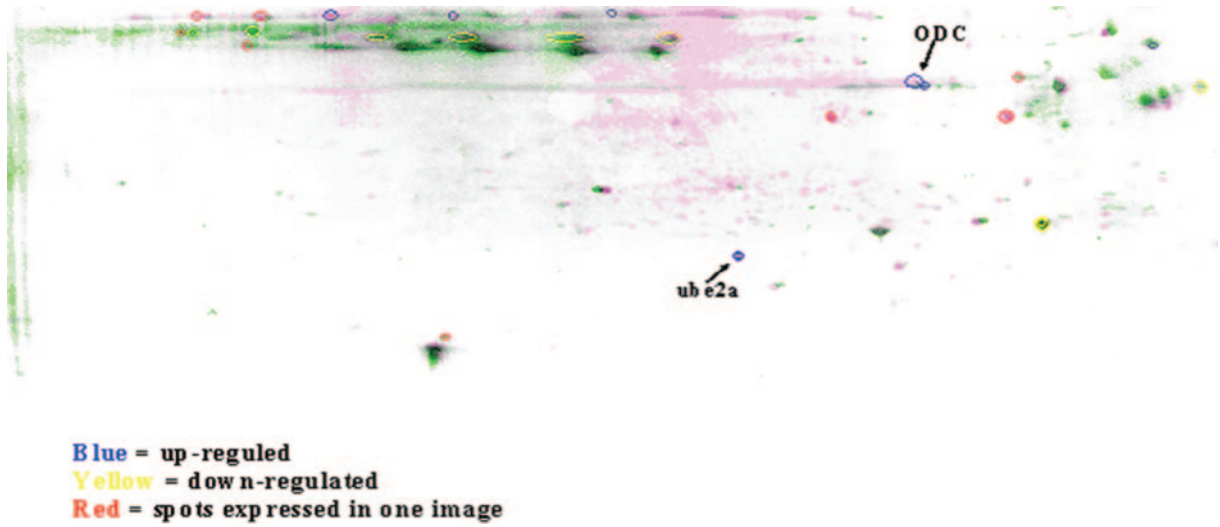

FIG. 6. **Differential display.** This differential display was created by Z3 software using the master gels from nicotine-treated and saline-treated controls. The software identified 21 proteins as being differentially expressed. Those spots circled with *blue* are proteins that were up-regulated by nicotine treatment; *yellow,* down-regulated; *green background circled with red*, newly expressed; and *magenta background circled with red*, no longer expressed.

TABLE III
Effect of continuous R-(+) nicotine infusion on the lung polyamine content of immunosuppressed rats

21-Day i.p. infusion dosage[a]	Polyamine		
	Putrescine	Spermidine	Spermine
	nmol (g wet weight)$^{-1}$ ± *S.D.*		
Saline	198 ± 14	759 ± 18	1533 ± 15
4 μg of nicotine kg^{-1} h^{-1}	288 ± 19	782 ± 22	1388 ± 32
400 μg of nicotine kg^{-1} h^{-1}	411 ± 33	820 ± 28	1420 ± 29

[a] $n = 3$.

19-fold increase, which is somewhat greater than the 14-fold increase detected by densitometry of the two-dimensional gels.

Effects of R-(+) Nicotine on Lung Polyamine Pools—If nicotine-mediated ODC up-regulation increases the rate of lung polyamine biosynthesis, increases in lung putrescine and polyamines could be expected. However, HPLC analysis (Merali *et al.* 1996b, 1999) (50, 53) of nicotine-treated and control lungs showed putrescine to be increased only 2-fold and spermidine and spermine not significantly altered (Table III). However, if there is a fine balance in AdoMet production and consumption, a relatively small increase in AdoMet consumption by a small increase in polyamine production could cause a marked reduction in AdoMet if production of AdoMet does not increase in response to demand; the lack of change in MAT activity supports this. It is also possible that increased polyamine production is masked by either enhanced polyamine cycling or enhanced polyamine export, and these possibilities were examined.

Effect of R-(+)-Nicotine on Polyamine Catabolic Enzyme Activity and Metabolic Flux of AdoMet—Polyamine cycling involves the catabolic loss of the AdoMet-derived aminopropyl group from a "higher polyamine" converting it to a "lower polyamine" followed by anabolic re-conversion to the higher polyamine. Although it is sometimes done in one step, catabolism usually takes two steps, one mediated by spermine-spermidine acetyl transferase (SSAT) and the other by acetylpolyamine oxidase (APAO). SSAT acetylates spermidine and spermine at N-1 and APAO cleaves the acetylated *N*-propyl group converting *N*-acetylated spermine to spermidine and *N*-acetylated spermidine to putrescine. If such catalysis is enhanced but polyamine concentrations are maintained at a steady level, putrescine and spermidine produced by this catabolism must be recycled back to spermidine and spermine, a process that consumes AdoMet. Alternatively, if acetylated polyamines are lost by diffusion through cell membranes as a consequence of being less polar, *de novo* polyamine biosynthesis must be increased to maintain polyamine concentrations, a process that also consumes AdoMet. To seek evidence for increased use of AdoMet by these pathways, we measured the content of *N*-1-acetylspermidine in lung extracts and the rate of production of *N*-1-acetylspermidine by dialyzed lung extracts incubated with the SSAT substrates spermidine and acetyl-CoA (50). Compared with saline-treated controls, lung homogenates from nicotine-treated animals had 11-fold more *N*-1-acetylspermidine and the dialyzed extract had a 4.5-fold greater rate of *N*-1-acetylspermidine production, evidence for enhanced polyamine catabolism (Table IV).

If nicotine treatment causes lungs to use more AdoMet for polyamine biosynthesis, then greater AdoMet DC activity would be expected and this was found. Dialyzed lung homogenates from nicotine-treated animals had 7 times greater AdoMet DC activity than homogenates from saline-treated controls (Table IV). In addition, there was a 6-fold increase in the concentration of methylthioadenosine, the byproduct of aminopropyl transfer from decarboxylated AdoMet for polyamine synthesis.

DISCUSSION

The data presented here demonstrate that intraperitoneal infusion of *R*-(+) nicotine into rats causes a significant accumulation of nicotine in the lungs and a 15-fold decrease in lung AdoMet but no change in liver or plasma AdoMet. These are similar to results obtained with guinea pigs (29) but with a lower nicotine dose. The data also show that nicotine treatment begun at the time of inoculation with *P. carinii* is strongly prophylactic against development of PCP in the rat model, results concordant with the statistically significant protective association found between smoking and PCP in a large clinical study with 476 patients (46). In another study involving 232 patients the authors stated: " . . . cigarette smoking was actually associated with an unexpectedly (and non-significantly, $p = 0.11$) decreased risk and slower time to development of PCP" (60). Results from a small study of 42 patients were interpreted as indicating that smoking doubles the risk of PCP; however, that study was compromised by a wide range in reported tobacco use in the control group and by 12 of the 15 PCP patients recently having had tuberculosis (61). We con-

TABLE IV
Effect of continuous R-(+) nicotine infusion on lung polyamine metabolism

Treatment was begun at the time the animals were inoculated with *P. carinii*. Animals were sacrificed 21-days post-inoculation and lung homogenates analyzed for enzyme activity and for polyamine metabolism intermediates.

21-Day i.p. infusion dosage[a]	Polyamine acetylation activity	*N*-1-Acetylspermidine content	AdoMet DC activity	Methylthioadenosine content
	pmol N-1-acetylspermidine (mg protein)$^{-1}$ h^{-1} ± S.D.	*pmol (mg protein)$^{-1}$ ± S.D.*	*pmol dcAdoMet (mg protein)$^{-1}$ h^{-1}) ± S.D.*	*pmol (mg protein)$^{-1}$ ± S.D.*
Saline	11.8 ± 4.1	72 ± 38	52 ± 20	123 ± 40
400 μg of *R*-(+)-nicotine kg^{-1} h^{-1}	52.3 ± 14	788 ± 124	377 ± 76	768 ± 58

[a] $n = 3$.

clude that, on balance, the clinical data indicate a protective association between cigarette smoking and PCP. Discovery of this association was remarkable considering that none of these studies included nicotine exposure a primary study parameter, smoking assessment was not controlled but taken from patient reports, and compounds delivered in cigarette smoke vary from brand to brand, from puff to puff and from smoker to smoker. When one considers that the lung irritation and damage caused by smoking most likely makes lungs more vulnerable to infection in general, this protective association is particularly striking. Our results with a rat model link the effect of smoking on PCP to nicotine and indicate that the proximal mechanism is depletion of lung AdoMet. This conclusion is supported by our observation that co-administration of AdoMet with nicotine reverses the effect. The importance of exogenous AdoMet for *Pneumocystis* is emphasized by the finding that AdoMet administration in the absence of nicotine enhances the development of PCP.

Because the effect of nicotine on lung AdoMet is chronic and takes weeks to develop (Fig. 2), it may involve a cascade of events culminating in depletion of lung AdoMet. While have no information on the early response to nicotine, we conclude the ultimate cause of lung AdoMet depletion is an increase in AdoMet consumption caused by increased polyamine metabolism. This conclusion is drawn from the following observations. Nicotine treatment does not change lung activity of MAT, the enzyme that produces AdoMet. Nicotine treatment does not cause an increase in lung tissue methylation index, which would be the case if there were an increase in consumption of AdoMet for methylation reactions, which ordinarily consume most AdoMet. Nicotine treatment does cause an increase in the lung the key polyamine biosynthesis regulatory enzymes ODC and AdoMet DC. Proteomic data show ODC protein is increased 14-fold and enzymatic data show ODC activity is increased 17-fold. Another laboratory reported a 2.2-fold increase in ODC activity in animals exposed to 10 min of 25% cigarette smoke each day for 8 weeks (62). AdoMet DC activity is elevated 7-fold and methylthioadenosine (the product of aminopropyl donation by decarboxylated AdoMet for polyamine synthesis) is increased 6-fold. Although putrescine, the product of ODC, is increased only 2-fold and the higher polyamines are essentially unchanged, the data show enhanced polyamine catalysis. Presumptive SSAT activity, the production of *N*-1-acetylspermidine by dialyzed lung extracts supplied acetyl CoA and spermidine, is increased 4.5-fold and the lung content of *N*-1-acetylspermidine is increased 11-fold. These data are consistent with depletion of lung AdoMet caused by nicotine treatment being driven by an increase in the catabolic/anabolic cycling of polyamines.

Because shifts in polyamine metabolism in response to stress are known, particularly increases in SSAT activity (47, 59), and nicotine treatment requires weeks to have full effect on lung AdoMet, the effect of nicotine on lung AdoMet may be part of a multistep stress response that culminates in changed polyamine metabolism. However, a previous study reported a 25-fold increase in lung ODC activity in animals 4 h after a dose of 5 mg nicotine kg^{-1} (57) so that it is also possible the primary effect is on polyamine pathways with AdoMet depletion being due to the cumulative effect of greater AdoMet consumption than production over weeks. Continued analysis of our proteomic data may shed light on this but a thorough examination will require a detailed time course study of the response of the proteome to nicotine treatment as well as time course studies of enzyme activities.

An interesting observation is that we did not find PCP to cause a reduction in lung AdoMet of the animals used for these experiments. Lungs of rats with PCP that were infused with saline contained 7.2 mg of AdoMet g^{-1} lung tissue (Table II), essentially the same as rats without PCP infused with saline, 7.3 mg of AdoMet g^{-1} lung tissue (Fig. 4). With a mean number of cysts in the lungs of 5.5 (10)^6 (Table II), these infections were not intense. In an unrelated experiment,[2] we did find a 50% reduction in the mean lung AdoMet in a group of 4 highly infected animals (mean cyst count of 10^9 per lung) compared with control uninfected animals. We also found a 50% reduction in the AdoMet of mink lung epithelial cell line Mv1Lu 96 h after a cell layer was inoculated with *P. carinii*.[2] Considering that PCP causes the plasma of rats (27) and patients (28) to become depleted of AdoMet, infusion of AdoMet enhances *P. carinii* infection in rats, moderate PCP does not cause a decrease in lung AdoMet and severe PCP causes only a 50% reduction in lung AdoMet, we conclude that *Pneumocystis* is limited in ability to extract AdoMet directly from host cells and must rely on the much lower amount of AdoMet in extracellular fluids. This emphasizes the vulnerability of this fungus to a limitation on AdoMet availability. It also leaves open a question regarding the importance of the lung specificity of nicotine and AdoMet if *P. carinii* does not extract this intermediate directly from lung cells. A reasonable explanation is that *Pneumocystis* relies on uptake of AdoMet from alveolar fluid, lung cells are the major source of this AdoMet and the AdoMet content of alveolar fluid drops when lung cell AdoMet drops. According to this hypothesis, the general depletion of plasma AdoMet caused by PCP would be driven by the AdoMet-depleted alveolar fluid serving as a sink for plasma AdoMet.

All of the work reported here was with the *R*-(+) isomer of nicotine and we have no data for the *S*-(−) isomer. However, work by others showed that *S*-(−) nicotine reduces both AdoHcy and AdoMet levels in the lung of guinea pigs (29). Interestingly, the *R*-(+) isomer had no effect on lung AdoHcy although it did cause a significant reduction in AdoMet (29). Conversely, liver AdoHcy was increased by the *R*-(+) isomer but not the *S*-(−), although neither affects liver AdoMet. The *R*-(+) isomer is a substrate for guinea pig lung azaheterocyle *N*-methyltransferase, an AdoMet-consuming reaction, while the *S*-(−) isomer competitively inhibits this enzyme. Both isomers cause an up-regulation of the polyamine synthesis regulatory enzyme ODC in lung tissue (57). Thus effects of the isomers on various pathways are different yet they both cause lung AdoMet to be reduced. This seems likely to be a fertile area for future investigation.

In conclusion, we show that nicotine acts prophylactically in

an animal model by reducing lung AdoMet via perturbation of polyamine metabolism. However, many issues remain to be resolved. Effectiveness in treating established disease in the animal model is unknown but the delayed effect on lung AdoMet suggests that nicotine may be better for prophylaxis than for treatment. The role of AdoMet in *Pneumocystis* metabolism and the reason for high consumption are not understood. We worked with the *R*-(+) epimer of nicotine and have no information on the effect of the natural *S*-(−) epimer found in plants. We cannot predict an effective human dose although the existing clinical data suggest that efficacy may be achievable with doses in the range of that delivered by cigarettes. Finally, there is a possibility that analogues of nicotine could have greater activity against PCP with less toxicity.

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