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 99.98% 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 PCP¹ (*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 synthesise 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
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 Km 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 contraction, 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 parasitic helminth diseases: 19 mg of nicotine kg−1 per os treats Fasciola, Taenia, and Ascaris infections (38). The IC50 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), demonstrating that helminths are treated effectively with nicotine. 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 Km is 14.2 μM (37).

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) (22) with the results of a broad clinical study reporting a correlation between smoking and recurrent PCP (46) (22) with the results of a broad clinical study reporting a correlation between smoking and recurrent PCP (46) (22) with the results of a broad clinical study reporting a correlation between smoking and recurrent PCP (46) (22) with the results of a broad clinical study reporting a correlation between smoking and recurrent PCP (46). From these associations, we developed the hypothesis that smoking produces nicotine analogues with useful activity, less addictive potential, and sulfamethoxazole ml−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 sternal bridge. The capillary was secured in place with cyanoacrylate surgical adhesive (VetBond). Incisions were closed with wound clips. The pump reservoir was accessed by the needle of a 26-gauge infusion set inserted into the fill port; the flexible tubing of the infusion set 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 of the manufacturer-reported delivery rate of approximately 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 tubing flushing out any of the remaining old solution through the drainage infusion set. The drainage 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, 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 was 0.5 nmol and limits of linearity were 0–100 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. Supernatant fractions were analyzed 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 extract. Supernatant fractions were 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 lungs were snap-frozen in liquid nitrogen and were crushed into a fine tissue sample using a mortar and pestle. The tissue sample was homogenized in 2 ml of 0.1 N HCL followed by centrifugation to remove the debris. The supernatant fraction was saved to be analyzed by HPLC. The tissue sample was homogenized in 2 ml of 0.1 N HCL followed by centrifugation to remove the debris. The supernatant fraction was saved to be analyzed by HPLC.

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powder using mortar and pestle. The powder was resuspended in 2 ml of buffer (150 mM KCl, 5 mM dithiotreitl, 5 mM HEPES, 5 mM MgSO4) and sonicated at 40 watts and 70% duty cycle for about 2 min, then centrifuged at 10,000 × g for 15 min at room temperature. The supernatant 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 (27). Preparative 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 N1-acetylated spermidine were analyzed by HPLC using Waters’ Aqueous 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 sonicated at 15 min for 4 °C in saline. Proteins were extracted from 500 mg of aliquot of lung tissue as follows. After freezing with liquid nitrogen, tissue samples were ground to a powder using a mortar and pestle, and with liquid nitrogen in the mortar. The frozen powder was added directly to 0.8 ml of cold lysis buffer (9.8 mM 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 fraction was used for Bradford protein assay (Bio-Rad). A 10% trichloroacetic acid and 20 μl 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 acetic/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 4 °C 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), 10% trichloroacetic acid and 20 μl 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 acetic/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 4 °C for 15 min, the supernatant was collected for two-dimensional protein electrophoresis.

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 mM urea, 30% glycerol, 2% SDS, 1% DTT, and 0.001% bromophenol blue in 0.5 mM Tris-Cl buffer, pH 8.8) (54) and then incubated for 15 min in 10 ml of equilibration buffer containing 250 μg 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) mounted in a Multiphor II electrophoresis unit. The gels were soaked for 15 min in 10 ml of cold 2× Laemmli sample buffer (equal parts 30 mM potassium ferricyanide and 100 mM sodium thiourea) 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 mg μl−1 with 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 2-aminocyano-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 450–2500; and low mass gate 400 Da. The laser wavelength was 337 nm and repetition was set at 1.5 Hz. The instrument produces a protonated ion, [M + H]+. 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 NCBI 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 supracapaculare 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 (r2 = 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 18 ± 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).
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
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 dexametha-
sone treatment (Fig. 2).

**Nicotine Treatment of an Animal Model of PCP**—We re-
peated the protocol with dexamethasone as above but inocu-
lated animals with *P. carinii* at the same time the *R*(+)-no-
tine 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.

**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.
Nicotine and Pneumocystis Pneumonia

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 | Mean cysts per lung × 10^9 (±S.E.) | Mean nuclei per lung × 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) |

*n = 3.*

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 | Plasma AdoMet µµ ± S.D. | Lung AdoMet µµ ± S.D. | Mean cysts per lung × 10^9 (±S.E.) | Mean nuclei per lung × 10^9 (±S.E.) |
|-----------------------------|-------------------------|-----------------------|------------------------------------|-----------------------------------|
| 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 (±2.8)           | 10.4 (±0.7)                        | 3.8 (±0.87)                       |

*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 is taken to reflect increased 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 consumption 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 immunsuppressed control rats treated with saline for 21 days and immunsuppressed 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 NCBI-nr 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 polyanine 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.
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-(+)/H11001 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-(+)/H11001 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...
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) 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, catalysis 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 catalysis 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 amidopropyl 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-
incidence 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 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 azaheterocycle N-methyltransferase, an AdoMet-consuming reaction, while the \(S(+)\) isomer competitively inhibits this enzyme. Both isoforms cause an up-regulation of the polyamine synthesis regulatory enzyme ODC in lung tissue (57). Thus effects of the isoforms 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

| Treatment | Polyamine acetylation activity | AdoMet DC activity | Methylthioadenosine content |
|-----------|-------------------------------|-------------------|-----------------------------|
|           | pmol N-1-acetylspermidine (mg protein\(^{-1}\) h\(^{-1}\) ± S.D.) | pmol dAdoMet (mg protein\(^{-1}\) h\(^{-1}\) ± S.D.) | pmol |
| 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.
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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.

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