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OCCURRENCE OF SPECIFIC STEROLS IN *Pneumocystis carinii*

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We have examined the sterol composition and biosynthesis of rat *Pneumocystis carinii*. We found a number of lipid components among the nonsaponifiable fraction that appear unique to *P. carinii*. These lipids are present in *P. carinii* purified preparations and *P. carinii*-infected whole lungs but absent from control lungs. They show chromatographic properties typical of sterols on GC and HPLC; they are present in free and esterified sterol fractions isolated by thin layer chromatography, they are precipitated by digitonin and they are labeled upon incubation of purified *P. carinii* cell suspensions with [^{3}H] mevalonate and [^{3}H] squalene. The finding of these sterols opens new opportunities for the identification of chemotherapeutic targets against *P. carinii*.

Pneumocystis carinii is an opportunistic pathogen with similarities to fungi (1) that poses a major threat to immunocompromised hosts (2). *P. carinii* is normally restricted to the lungs and it can cause severe pneumonia that may result in death. The AIDS epidemic puts increasing numbers of people at risk for *P. carinii* infections. In addition, a complicating element is that this type of patients has less tolerance for the most effective presently available drugs against this parasite (3). These facts create an urgent need for the development of new chemotherapeutic approaches.

Sterol biosynthesis is an important target for chemotherapy of mycoses. A number of drugs are available that affect different steps in the biosynthesis of fungal sterols. Among them, imidazole derivatives, like ketoconazole and itraconazole, inhibit the cytochrome P-450-dependent 14-alpha demethylation of lanosterol (4). Another step, the one catalyzed by squalene epoxidase, is the target of two types of antimycotics: allylamines and tolnaftate (5). Blockade at an even earlier step is possible and inhibition by Compactin of hydroxymethylglutaryl-CoA reductase can also prevent yeast proliferation (6). In addition,

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the fungal sterols themselves also seem to be chemotherapeutic targets on their own. Thus, ergosterol interacts directly with polyene antifungals, like amphotericin B and nystatin (7).
In this work, we have examined the sterols of *P. carinii* and aspects of their metabolism. We have used a rat model with numerous advantages over previous systems. We show for the first time the occurrence of a peculiar sterol composition in *P. carinii* and present evidence of sterol biosynthetic activity in this organism.

Methods

***P.carinii* cell preparations:** For the production of *P. carinii*, our model was based on that described by Boylan and Current (8). Briefly, viral antigen-free rats, weighing 130-150 g (Harlan Sprague Dawley Indianapolis, IN) were housed in sterile filtered air facilities and were fed autoclaved balanced rat food and hyperchlorinated water. They were immunosuppressed by an initial subcutaneous injection of 30 µg/kg methylprednisolone (Depo-Medrol, The Upjohn Co., Kalamazoo, MI), followed by weekly infections at 20 µg/kg. After one week of the first corticosteroid injection, the rats were mildly anesthesized by short exposure to 70% CO_2 and intratracheally inoculated with 10^7 organisms/rat. Two days later, the rats were inoculated for a second time to ensure proper infection. After 6-8 weeks, the animals were killed by exposure to Halothane (Halocarbon Laboratories, North Augusta, SC) and their lungs were removed. Immunosuppressed, non inoculated rats, kept under the same conditions, did not develop *P. carinii* or other infections and were killed after the same time period to obtain control preparations. The lungs were rinsed in 25 mM Hepes/1.8 mM $CaCl_2$/150 mM NaCl, pH 7.4 (HCS buffer) and placed in a Petri dish containing the same buffer. Lung smears were prepared, heat-fixed and stained with Diff-Quik (Baxter Chemical Co., Deerfield, IL). The level of infections was determined by counting *P. carinii* nuclei under light microscopy at X 1,000 magnification. We routinely obtained high *P. carinii* infections (rated 4 in a 0-4 scale) with no contamination with bacteria or fungi. The lungs excised from uninoculated (control) and experimentally infected rats were freed from non lung tissues and minced in a solution containing 25 mM Hepes/1.8 mM $CaCl_2$/ 2 % N-acetyl-cysteine, pH 7.4, then dissociated in a Stomacher lab blender 80 (Tekmar Cincinnati, OH) for 10 min, at room temperature. N-acetyl-cysteine was included to take advantage of its mucolytic properties. The resulting suspension was passed through a cell dissociation sieve (40 mesh) (SIGMA Chemical Co., St. Louis, MO) and the sieve then washed with HCS buffer to a final sievate volume of 50 ml. The sievate was centrifuged at 925 g for 10 min (high spin), at 4°C, in a swinging bucket rotor of a IEC PR-2 centrifuge (IEC, Needham Heights, MA). The pellet was resuspended in 10 ml of 0.85 % ammonium chloride, pH 6.8, vortex agitated and incubated at 37°C for 15 min to lyse erythrocytes and host cells. After this period, 3 volumes of HCS buffer were added and the pellets were collected by high spin centrifugation, resuspended in HCS buffer and centrifuged at 60 g for 10 min (low spin) at 4°C. The resultant low spin supernate was then subjected to a high spin and that pellet processed through another cycle of low- and high-spin centrifugation. The final pellet was resuspended in 5 ml HCS buffer and the suspension filtered through an 8 µ polycarbonate membrane (Nucleopore, Pleasanton, CA). Purified *P. carinii* preparations, observed under phase contrast microscopy, showed large amounts of cysts and a small number of isolated host nuclei. The latter were the only elements found in samples prepared from control lungs. Trophozoites and cysts were observed in the *P. carinii* preparations by fluorescence microscopy after labeling with calcein AM (9). Our samples contained 70-80% living cells, as determined using calcein AM in combination with propidium iodide (Molecular Probes, Eugene, OR) as vital stains (9). Cell numbers in the final *P. carinii* preparations were determined on diluted aliquots stained with Diff-Quik.
Lipid analyses: Lipids from freshly isolated *P.carinii* in HCS buffer were extracted according to Bligh and Dyer (10). In some cases, lipids from whole lungs of infected and non-infected (control) immunosuppressed rats were extracted using the same method. This

procedure allowed us to obtain samples containing the lipids of all forms of *P. carinii* life stages present in the lung without possible alterations due to the purification procedure. Owing to the massive infections present in the lungs, *P. carinii* typical lipids were easily detected by comparison with the non infected controls. Thin layer chromatography was carried out as described before (11). Saponification was carried out as previously described (12). Digitonin precipitation was performed according to Kates (13).The original sample, the precipitate and the supernatant were analyzed by GC and HPLC as described below. High performance liquid chromatography (HPLC) was carried out on a Gilson Medical Elec. equipment (Middleton, WI) or on a Beckman 100A (Beckman Instruments, Fullerton, CA) using a 250 x 4.6 mm column packed with 5 μm ODS. Lipids, monitored at ABS_{210nm}, were eluted with methanol at 1 ml/min and 0.5 ml fractions were collected. Gas chromatography (GC) was carried out on a Hewlett Packard 8530A gas chromatograph (Palo Alto, CA), at 250°C, using a 6ft glass column packed with 3% OV-17 and a flame ionization detector. N_2 was the carrier gas.

Incubation with radiolabeled sterol precursors: The purified *P. carinii* and control samples were high spin centrifuged and resuspended in 5 ml of incubation medium, consisting of Dulbecco's modified essential medium (DMEM), 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (all from SIGMA Chemical Co.), containing 10-50 μCi [^{14}C] sodium acetate, 100 μCi [^{3}H] mevalonic acid (both from Amersham, Arlington Heights, IL) or 2 μCi [^{3}H] squalene (New England Nuclear, Boston, MA). The solvents of the radioactive compounds were first evaporated under N_2. [^{14}C] acetate and [^{3}H] mevalonic acid were then directly dissolved in the incubation medium. [^{3}H] squalene was dissolved in 5 μl of dimetyl sulfoxide and then added to the medium. Incubation was carried out at 37°C in a 5% CO_2, humidified atmosphere. After 24 h, the suspensions were centrifuged and washed twice with HCS buffer. The lipids were extracted and saponified. The nonsaponifiable lipids were separated by HPLC as described above.The radioactivity in each fraction was determined on a Beckman 1001 liquid scintillation spectrometer.

Results and Discussion

The analysis by HPLC of nonsaponifiable lipids from purified *P. carinii* preparations revealed the presence of five major peaks with retention times typical of sterols. By contrast, identically processed samples from control animals showed only one major peak which co-chromatographed with cholesterol, suggesting that *P. carinii* contains peculiar sterols absent from the host (Fig. 1). To further explore this possibility, we employed gas chromatographic analysis (GC). The profiles obtained using nonsaponifiable lipids extracted from whole lungs of control and *P. carinii* -infected animals also showed conspicuous differences, as shown in Fig. 2 where two peaks found only in the infected lungs are marked A and B. These peaks were also present in thin layer chromatographic fractions corresponding to free sterols and esterified sterols of *P.carinii* preparations analyzed by GC (not shown).

To investigate the nature of the peaks obtained by GC and HPLC, we subjected the samples to a digitonin treatment that precipitates sterols and analyzed the results by GC. This treatment essentially precipitated all compounds with retention times between 10 and 30 min (not shown), further substantiating the sterol nature of the compounds peaking in this region. Analysis by HPLC also showed that the five peaks marked I to V in Fig. 1 are precipitated by digitonin (not shown).

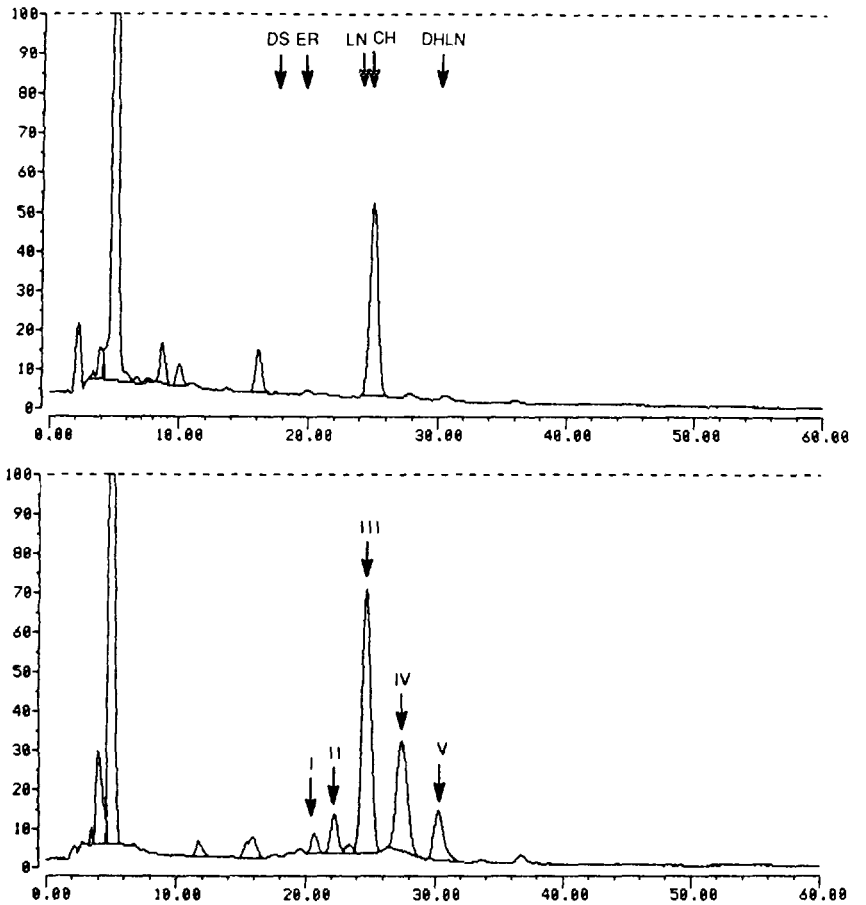

Fig. 1. HPLC elution profiles of nonsaponifiable lipids from control (upper tracing) and *P. carinii* (lower tracing) purified preparations. Ordinate is absorbance at 210 nm (100 = 0.2 A_{210} units). *P. carinii* cells were purified from the lungs of immunosuppressed infected rats as described in Methods. Control samples consisted of identically processed lungs from immunosuppressed non infected rats. The arrows at the top of the upper tracing show the positions at which different markers peak: DS: desmosterol; ER: ergosterol; LN: lanosterol; CH: cholesterol; DHLN: dihydrolanosterol. The roman numbers I-V identifying peaks in the lower tracing are those used in Table 1 and in the text. Similar profiles were obtained in five different experiments.

Since the GC procedure results in an incomplete resolution of the peaks and also because of the possibility that the different HPLC peaks could contain more than one compound, we employed a combination of these two procedures to examine the sterols of *P. carinii*. We used the ODS HPLC column to chromatograph nonsaponifiable lipids from whole control and infected lungs and collected all fractions of the eluates. Each fraction was concentrated and analyzed on GC after addition of a known amount of cholestane as an internal standard. Using this procedure, we resolved 12 different compounds in *P. carinii* -infected lungs and quantitated them on the basis of their FID response compared to that of cholestane (Table 1). Of these, six are also present in non infected lungs and can, therefore, be attributed to the host. It is noteworthy that important differences exist between control and infected lungs with respect to the relative amounts of the sterols they have in common. Except for cholesterol, all

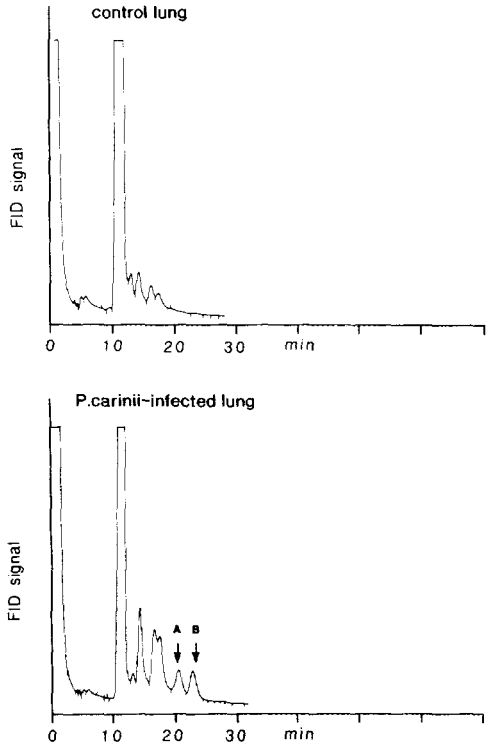

Fig. 2. Gas chromatograms of nonsaponifiable lipids from control and *P. carinii* -infected whole lungs. Note the two peaks marked A and B that are only present in the sample containing *P. carinii.* Similar profiles for control and infected lungs were obtained in two other separate experiments.

of them are increased in the infected lungs. The two *P. carinii* peaks A and B observed in the direct GC analysis of total nonsaponifiables (Fig. 2) correspond to components 9 and 10 (peak A) and 11 (peak B). When larger samples than the ones corresponding to the figure shown were analyzed, an additional peak was detected with larger retention time. This is designated as component 12 in Table 1.

In another set of experiments, we examined the incorporation by *P. carinii* cell suspensions of radiolabeled precursors that enter the sterol biosynthetic pathway at different levels. We found that while [^{14}C] sodium acetate was not incorporated into *P. carinii* lipids (not shown), [^{3}H] mevalonic acid (MVA) and [^{3}H] squalene (Sq) were taken up and converted to sterols by these cells. Fig. 3, a and b, show the HPLC elution profiles of radioactivity resulting from incubation of *P. carinii* purified cell suspensions with [^{3}H] MVA and [^{3}H] Sq, respectively. MVA was incorporated into all the peaks described in Fig. 1 (I-V). If the cells were homogeneized before addition of the precursor, no incorporation of MVA was observed, indicating that *P. carinii* cell integrity is required for the conversions observed. By contrast, a control preparation purified from a non infected lung did not incorporate [^{3}H] MVA into sterols. Labeling with [^{3}H] Sq produced a more complex pattern than that obtained with [^{3}H] MVA. Four of the peaks (I, III, IV and V) were labeled. These results provide additional evidence indicating that this cell is capable of its own sterol biosythesis.

Table I: Sterol components of control (immunosuppressed, non infected) and *P.carinii*-infected whole lungs. Nonsaponifiable lipids were first separated on HPLC and each fraction was then analyzed by GC as described in Methods. Columns 2 and 3 indicate the retention times relative to those of cholesterol (RRT_{chol}) on GC and HPLC, respectively. Component 1 is cholesterol. Column 4 shows the correspondance of each peak with the peaks marked I-V in Fig. 1. Columns 5 and 6 report the relative amounts of each compound as determined by GC-FID using cholestane as internal standard. Note that each compound is identified by its retention time on GC and HPLC and that six of the listed components are only present in *P. carinii* - containing preparations. They are marked with * in column 1. We obtained similar results from two different samples examined in the same way.

	1 Comp. number	2 GC RRT_{chol}	3 HPLC RRT_{chol}	4 HPLC peak #	5 control %	6 P. carinii %
	1	1	1	III	94.17	70.42
*	2	1.08	0.80	I	0	1.01
	3	1.20	0.83	I	0.29	3.76
	4	1.33	1.01	III	2.04	5.25
	5	1.40	0.80	I	1.28	6.09
	6	1.56	1.05	IV	0.35	3.39
*	7	1.60	0.80	I	0	3.13
	8	1.63	1.20	V	1.87	2.29
*	9	1.83	0.90	II	0	1.42
*	10	1.87	1.13	V	0	1.91
*	11	2.15	1	III	0	1.31
*	12	2.62	1.20	V	0	0.02

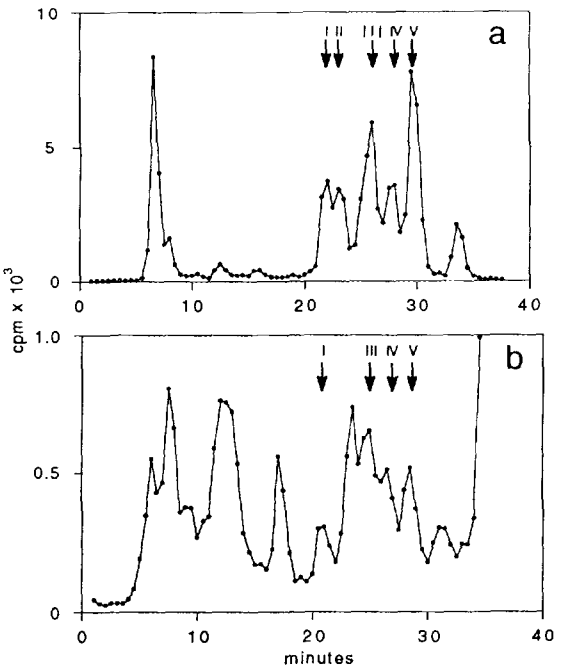

Fig. 3. Incorporation of a) [^{3}H] mevalonic acid and b) [^{3}H] squalene into nonsaponifiable lipids from purified *P. carinii* cell suspensions. After 24 h of incubation with the precursors, the lipids were extracted, saponified and chromatographed on an ODS HPLC column as described in Methods. The figure shows the distribution of radioactivity in the eluate fractions. Roman numbers designate the positions of the peaks identified in Fig. 1. The results are representative of two different experiments.

In conclusion, this work is the first report in which a peculiar sterol composition and sterol biosynthetic capabilities in *P. carinii* are presented. It opens new avenues in the search of chemotherapeutic targets in this cell. The methods presented here can be directly employed to compare the effect of different inhibitors of sterol biosynthesis. Such comparisons may offer valuable clues to the design of new drugs.

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