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# Total Cellular Fatty Acid Composition of Cultured *Pneumocystis carinii*

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**Oleic acid makes up >50% of the total fatty acids of *Pneumocystis carinii* grown on WI-38 cells. Oleic acid levels increased in parallel with increasing trophozoites over 7 days in culture. The fatty acid composition of *P. carinii* resembles that of certain fungi but differs from those of lung surfactant lipid, host cells, and fetal bovine serum.**

Metabolic studies of *Pneumocystis carinii* have most often used *P. carinii* separated from infected lung tissue, but contaminating substances in such preparations prevent absolute differentiation of attributes of the organism from those of the host (21, 32). A short-term culture method (5), dependent on the presence of feeder cells and fetal bovine serum (FBS), does support proliferation of *P. carinii* in a controlled environment, but growth in a continuous culture system is not possible.

*P. carinii* trophozoites obtained directly from rat lungs are reported to be coated with lung surfactant and lung surfactant protein A (21, 32). Rat lung surfactant is a complex of protein and lipid, of which surfactant protein A and phosphatidylcholine are the major components (24, 29). The phosphatidylcholine portion of rat surfactant lipid contains 85% of the fatty acids as palmitic acid (24). In the culture system, major lipid sources are FBS, feeder cells, and adherent lung surfactant and *P. carinii*. The lipid composition of FBS (7, 12, 27) is vastly different from that reported for rat lung surfactant (24) but similar to the WI38 cell composition reported herein.

The fatty acid composition of *P. carinii* free of lung surfactant and host tissue fragments has not been reported. In the present study, we examined the fatty acid composition of cultured *P. carinii* to better understand *P. carinii* lipid metabolism, develop better culture conditions, and compare the fatty acid composition of *P. carinii* with those of closely related eukaryotes.

All organic solvents were obtained from Burdick and Jackson (Baxter Scientific, Chicago, Ill.) and were of the highest grade available. Acetyl chloride was purchased from Aldrich Chemical Co., Milwaukee, Wis.

Cultures of *P. carinii* grown on WI-38 cell monolayers were prepared and quantitated for growth as previously described (4). The supernatant containing *P. carinii* was pooled from four identical wells (24-well plates). Samples were obtained on days 1, 3, 5, and/or 7 of culture by removal of 0.4 ml from each well following gentle agitation of the medium in each well to suspend the *P. carinii* but not disturb the cell monolayer. *P. carinii* cells were harvested by centrifugation for 5 min in a Beckman model E microcentrifuge. The pellets were suspended in either sterile saline and or phosphate-buffered saline and pelleted twice by centrifugation to remove the medium. Total lipid fatty acid methyl esters were obtained directly from the pellet (16). Briefly, each pellet was suspended in 100 μl of high-pressure liquid chromatography grade water, and the contents were transferred to culture tubes (13 by 100 mm). Each centrifuge tube was rinsed three times with 200 μl of methanol, which was in turn added to the culture tube. Benzene, 400 μl, was added to the tube, followed by 1 ml of freshly prepared 5% acetyl chloride in methanol. Each tube was briefly gassed with $N_2$ and tightly sealed with a Teflon-lined screw cap. The tubes were heated in boiling water for 1 h. After cooling, 1 ml of water and 1 ml of hexane were added, the tube was capped, and the contents were mixed by vortexing. Following 5 min of centrifugation at 1,800 × *g*, the hexane layer was removed to an injection vial and evaporated under a stream of $N_2$. The extraction and evaporation steps were repeated. Finally, the sample was dissolved in 100 μl of isooctane, the vial was crimp sealed with a Teflon-lined cap, and the contents were subjected to automated gas-liquid chromatography.

Samples of monolayers of WI-38 cells were grown on sterile glass coverslips placed in the bottoms of culture wells (21). After 7 days of culture with or without inoculation with rat lung homogenate containing *P. carinii*, the coverslips were removed and rinsed in three changes of phosphate-buffered saline. The cell monolayer lipids were then esterified directly on glass coverslips as described above. Total lipids in the cell culture supernatant (100 μl) were esterified directly (16).

Samples of fatty acid methyl esters were separated by gas-liquid chromatography on a methyl phenyl silicone fused silica capillary column (25 m by 0.2 mm [inside diameter]; Hewlett-Packard, Avondale, Pa.) in a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector, a 3392A integrator, and a 7673A autosampler. The system controller was a Hewlett-Packard 9000-300 series computer operating under Microbial Identification software (Microbial I.D., Inc., Newark, Del.). Calibration standards were run automatically every 10th sample.

The fatty acid composition of the pellets from proliferating cultures and nonproliferating cultures treated with trimethoprim-sulfamethoxazole (TMPX) was determined for days 1, 3, 5, and 7 of culture (Table 1). In samples from wells containing proliferating culture, a steady decline in palmitic acid (16:0) and a steady rise in oleic acid (18:1) were observed. The TMPX-inhibited culture samples remained

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TABLE 1. Total fatty acid composition of *P. carinii* containing pellets from proliferating and TMPX-treated cultures

| Culture | Fatty acid(s) | % of total fatty acids on: | | | |
|---|---|---|---|---|---|
| | | Day 1 | Day 3 | Day 5 | Day 7 |
| Proliferating | 14:0 | 2.7 | 2.1 | 2.5 | 2.4 |
| | 16:0 | 40.4 | 35.8 | 33.6 | 32.2 |
| | 16:1 | 4.7 | 3.5 | 2.4 | 2.7 |
| | 18:0 | 11.8 | 14.4 | 15.0 | 15.8 |
| | 18:1 | 18.5 | 25.2 | 26.1 | 31.1 |
| | 18:2 | 4.0 | 3.6 | 3.3 | 2.8 |
| | 20:4 | 5.0 | 5.1 | 6.4 | 5.1 |
| | DMA[a] | 4.6 | 3.9 | 3.8 | 3.0 |
| | Others | 8.4 | 6.3 | 7.0 | 4.9 |
| TMPX treated | 14:0 | 3.0 | 2.9 | 2.8 | 3.9 |
| | 16:0 | 35.6 | 37.9 | 38.2 | 38.9 |
| | 16:1 | 6.1 | 3.9 | 2.6 | 1.3 |
| | 18:0 | 11.9 | 13.4 | 13.8 | 13.7 |
| | 18:1 | 18.9 | 20.6 | 20.2 | 20.6 |
| | 18:2 | 4.1 | 3.9 | 3.6 | 3.1 |
| | 20:4 | 6.7 | 5.6 | 7.4 | 6.0 |
| | DMA[a] | 4.9 | 4.3 | 4.1 | 7.2 |
| | Others | 8.7 | 7.6 | 7.3 | 5.3 |

[a] DMA represents the total 16- and 18-carbon DMA.

constant, except for a decline in the relative percentage of palmitoleic acid (16:1). The percentage of 18:1 in the total lipids of the pellet containing *P. carinii* parallels the increased trophozoite count over 7 days of culture (Fig. 1). Addition of TMPX, which prevented proliferation of *P. carinii* (2), also abolished the increase in 18:1 in the total lipid from the pellet.

The fatty acid composition of WI-38 cells at day 7 of incubation with or without the *P. carinii* inoculum was also determined (Table 2). Except for a small but significantly

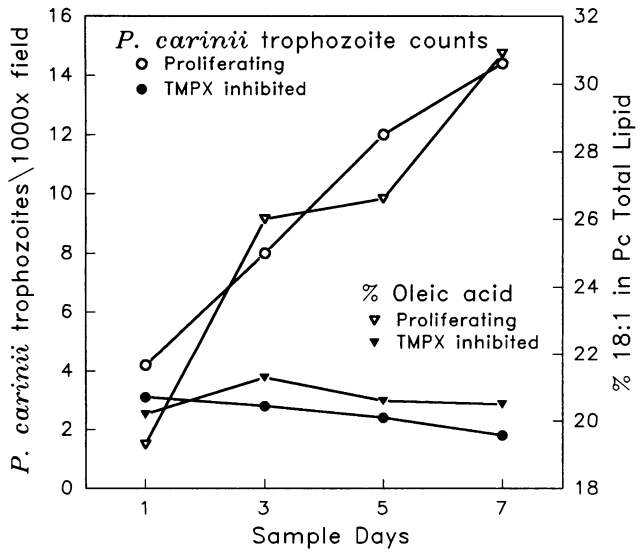


FIG. 1. Dual plot of *P. carinii* (Pc) trophozoite counts and oleic acid percentage of total lipids of pellets from culture supernatant versus days of culture. The oleic acid values on the right abscissa begin at 18% since that reflects the approximate levels of oleic acid in cell culture supernatant and WI-38 cells in the absence of *P. carinii*.

TABLE 2. Fatty acid composition of WI-38 cells without and with *P. carinii* inoculum

| Fatty acid(s) | Mean % of total fatty acids in WI-38 cells ± SD | |
|---|---|---|
| | Uninoculated ($n$ = 3) | Inoculated ($n$ = 3) |
| 14:0 | 0.9 ± 0.09 | 1.2 ± 0.19 |
| 16:0 | 18.8 ± 0.44[a] | 22.9 ± 0.29[a] |
| 16:1 | 3.4 ± 0.11 | 2.3 ± 0.49 |
| 16:0 DMA | 6.7 ± 0.08 | 7.5 ± 0.30 |
| 18:0 | 16.9 ± 0.34 | 15.2 ± 0.33 |
| 18:1 | 17.9 ± 0.03 | 17.7 ± 0.64 |
| 18:2 | 4.1 ± 0.56 | 5.4 ± 1.66 |
| 18:1 DMA | 2.7 ± 0.21 | 1.6 ± 0.21 |
| 18:0 DMA | 3.7 ± 0.11 | 3.1 ± 0.20 |
| 20:4 | 14.9 ± 0.54 | 14.5 ± 0.26 |
| Others | 10.0 ± 1.06 | 8.5 ± 0.70 |

[a] Significantly different ($P < 0.05$).

greater percentage of 16:0 in cells that received the lung homogenate containing *P. carinii*, there appeared to be no difference. The increase of 16:0 could reflect lung surfactant in the inoculum; lung surfactant is rich in 16:0 (24). WI-38 cells contain significant amounts of 16- and 18-carbon dimethylacetals (DMA) (16:0 DMA, 18:1 DMA, and 18:0 DMA in Table 2) derived from plasmalogens, which is characteristic of cultured human fibroblasts (25). However, no significant amounts could be attributed to *P. carinii*. The fatty acid composition of the cell culture supernatants from both proliferating and TMPX-treated wells reflected the fatty acid composition of the total lipids of FBS (27) and remained constant throughout the course of these studies.

The material pelleted from the supernatant of control and drug-treated wells contained, in addition to *P. carinii* lipid, lipid from at least two additional sources: residual lipid from the rat lung homogenate used to inoculate the culture and rare WI-38 cells sloughed into the medium. To assess the contribution of this material, we ran parallel analyses of control and drug-treated samples and examined the areas under the gas-liquid chromatography peaks (integrator values) from which the percentages of the major fatty acids (myristic acid [14:0], 16:0, 16:1, stearic acid [18:0], 18:1, linoleic acid [18:2], arachidonic acid [20:4], total DMA, and others) were calculated. Table 3 represents the fatty acid percentages and peak areas on day 7 of study A (Table 1) and a separate study (study B). Since care was taken to prepare each sample in an identical and quantitative manner, the peak areas of the fatty acids of the control samples were reduced by the peak areas of the TMPX-treated wells to obtain the fatty acid composition attributable to *P. carinii*. The most striking aspects of these data are the high percentage of 18:1 in *P. carinii* total lipid and the low levels of polyunsaturated fatty acids.

The in vitro culture method has been used successfully to predict the in vivo activity of antifolates (21), 8-aminoquinolines (4, 23), and inosine analogs (3, 21–23, 26), suggesting that cultured *P. carinii* is metabolically active over short periods of time. The study results reported herein expand our understanding of lipid metabolism in cultured *P. carinii*. The fatty acid composition of cultured *P. carinii* does not reflect that of the host cells, FBS lipids, or rat lung surfactant. The fatty acid composition of *P. carinii* on day 1 of culture was similar to that reported for *P. carinii* obtained from a series of microfiltrations (14). However, by day 7 of

TABLE 3. Fatty acid composition and integrator values at day 7

| Study and fatty acid(s) | Area under GLC[a] peak, % of total fatty acids | | |
|---|---|---|---|
| | Proliferating culture | TMPX-inhibited culture | Proliferating minus TMPX |
| A | | | |
| 14:0 | 3,217, 2.4 | 3,536, 3.9 | −319, −0.7 |
| 16:0 | 43,777, 32.2 | 35,183, 38.9 | 8,594, 18.8 |
| 16:1 | 3,703, 2.7 | 1,162, 1.3 | 2,541, 5.6 |
| 18:0 | 21,507, 15.8 | 12,438, 13.7 | 9,069, 19.9 |
| 18:1 | 42,275, 31.1 | 18,666, 20.6 | 23,609, 51.8 |
| 18:2 | 3,838, 2.8 | 2,813, 3.1 | 1,025, 2.2 |
| 20:4 | 6,976, 5.1 | 5,423, 6.0 | 1,553, 3.4 |
| DMA | 4,104, 3.0 | 6,519, 7.2 | −2,415, −5.2 |
| Others | 6,711, 4.9 | 4,765, 5.3 | 1,946, 4.3 |
| Total | 136,108, 100.0 | 90,505, 100.0 | 45,603, 100.1[b] |
| B | | | |
| 14:0 | 2,619, 1.1 | 3,695, 2.1 | −1,076, −2.1 |
| 16:0 | 66,267, 28.7 | 63,748, 35.5 | 2,519, 4.9 |
| 16:1 | 4,824, 2.1 | 2,536, 1.4 | 2,288, 4.5 |
| 18:0 | 36,392, 15.8 | 26,349, 14.7 | 10,043, 19.6 |
| 18:1 | 89,488, 38.8 | 48,832, 27.2 | 40,656, 79.5 |
| 18:2 | 6,994, 3.0 | 7,020, 3.9 | −26, −0.1 |
| 20:4 | 9,453, 4.1 | 11,190, 6.2 | −1,737, −3.4 |
| DMA | 6,104, 2.6 | 5,483, 3.0 | 621, 1.2 |
| Others | 8,611, 3.7 | 10,770, 6.0 | −2,159, −4.2 |
| Total | 230,752, 99.9[b] | 179,623, 100.0 | 51,129, 99.9[b] |

[a] GLC, gas-liquid chromatography.
[b] The total percentage does not equal 100% because of rounding.

culture, >90% of the fatty acids of *P. carinii* were monoeneoic or saturated (Table 3). The differences observed may represent shedding and/or metabolism of surfactant protein and lipid by *P. carinii*. Surfactant protein A and surfactant lipid avidly bind to *P. carinii* (21, 32). The presence of surfactant lipid would be expected to substantially affect fatty acid composition because of the presence of large amounts of disaturated phosphatidylcholine (21).

Growth of *P. carinii* is not limited to the lungs of humans (13). We have observed *P. carinii* microscopically in stained sections of the spleens and livers of infected rats (1). Thus, *P. carinii* does not appear to have an absolute requirement for the unique lipid environment of the lungs for growth.

Analysis of *P. carinii* total lipid fatty acids can contribute to understanding of the taxonomy of this organism. While identification of fungi by total cellular fatty acid analysis has not been as successful as that of bacteria, major group separations based on degrees of unsaturation and chain lengths of fatty acids are possible (6, 18–20, 30). Many reports have linked *P. carinii* to the fungi on the basis of rRNA (8, 28), surface carbohydrates (17), and the enzyme organization and amino acid composition of an enzyme (9). The fatty acid composition of *P. carinii* reported here most closely resembles that of *Schizosaccharomyces pombe* (high 18:1 and low 16:1 levels) (15, 31) and qualitatively resembles that of *Saccharomyces cerevisiae* (6, 20), some *Candida* species (30), and *Torulopsis glabrata* (18) but is unlike that of other fungi and protozoans (10). Any single chemotaxonomic measurement can lead to inconsistencies (11); however, examination of several parameters simultaneously has improved the usefulness of chemotaxonomy (11, 18, 30). Our data complement those cited above and support the conclusion that *P. carinii* is closely related to the fungi.

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