Inhibition of growth of *Pneumocystis carinii* by lactoferrins alone and in combination with pyrimethamine, clarithromycin and minocycline

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The *in vitro* activity of lactoferrins alone and in combination with clarithromycin, minocycline and pyrimethamine was investigated against three clinical isolates of *Pneumocystis carinii*. Susceptibility was tested by inoculating isolates on to cell monolayers and determining the parasite count after 72 h incubation at 37°C. The culture medium was supplemented with serial dilutions of each agent. At 20 mg/L, bovine lactoferrin, the most active agent, suppressed the growth of cystic and trophic forms by >60%. Human lactoferrin, at the same concentration, suppressed the growth of cystic and trophic forms by >50%. Lactoferrins at 20 mg/L combined with clarithromycin 4 mg/L had high anti-*P. carinii* activity, with a >90% decrease in cystic and trophic form counts. Our study suggests that lactoferrins may inhibit *P. carinii* growth *in vitro* and act synergically with other clinically used compounds. These findings lend experimental support to the use of iron-chelating agents in the therapy of pneumocystis infections.

Materials and methods

**Parasite preparation and cell culture**

Three clinical isolates of *P. carinii* were obtained from bronchoalveolar lavages of three immunocompromised patients who had not received prior anti-*P. carinii* therapy.

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Specimens were combined, homogenized in an equal volume of phosphate-buffered saline (PBS) by forced passage through a thin needle in order to disaggregate the clumps, and then filtered through sterile gauze. The homogenate was centrifuged (1800 g for 15 min) and the pellet resuspended in physiological saline. Contaminating bacteria were partially eliminated by four washes in 10 mL of physiological saline followed by incubation in phosphate-buffered saline containing ampicillin (2000 mg/L) and streptomycin (2000 mg/L) for 4 h at 37°C. Organisms were pelleted by centrifugation (1800 g for 20 min), resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (BioWhittaker, Walkersville, MD, USA), stained with methanamine silver (to detect cystic forms) or Giemsa stain (to detect P. carinii nuclei) and finally aliquoted for culture. The final inoculum was $5 \times 10^{3}$ P. carinii organisms/mL. Viability was assayed by double staining with fluorescein diacetate (FDA) (Sigma–Aldrich, Milan, Italy) and propidium iodide (Sigma–Aldrich). Briefly, the organisms were resuspended in 0.1 mL of PBS. After addition of 0.1 mL of FDA 40 mg/L and 0.03 mL of propidium iodide 20 mg/L and incubation at room temperature for 5 min, the incubation mixtures were further diluted with an equal volume of PBS and analysed by flow cytometry.

The human lung epithelial cell line A-549 (BioWhittaker) was maintained in 25 cm$^2$ tissue culture flasks. Medium consisted of DMEM (BioWhittaker) containing 0.10 mg/L Fe(NO$_3$)$_3$ with 10% fetal calf serum (BioWhittaker), 1% L-glutamine (BioWhittaker), 20 mM N-2-hydroxyethylpiperazine-N-ethanesulphonic acid (HEPES) (Sigma–Aldrich), penicillin G 100 U/mL, streptomycin 100 mg/L and amphotericin B 0.5 mg/L. Cells were detached from the surface of flasks using 0.25% trypsin and 0.53 mM EDTA in PBS; then they were counted using a haemocytometer.

Drugs

Compounds were all purchased from Sigma–Aldrich, except clarithromycin (Abbott, Rome, Italy). Iron-free human lactoferrin (lactoferrin H) and iron-free bovine lactoferrin (lactoferrin B) were dissolved in DMEM. Trimethoprim, pyrimethamine and clarithromycin were dissolved in methanol/acetone (1:1) at a concentration of 1 mg/mL. Sulphamethoxazole was dissolved in dimethylsulphoxide at 1 mg/mL. Minocycline was dissolved in distilled water at 1 mg/mL. Solutions of drugs were made fresh on the day of assay or stored at –80°C in the dark for short periods.

Susceptibility testing

Serial dilutions of each drug were prepared in DMEM. All drugs were tested at concentrations close to that which could be achieved clinically. Lactoferrins H and B were tested at concentrations of 1, 10 and 20 mg/L, clarithromycin and minocycline at 1, 2 and 4 mg/L and pyrimethamine at 0.1, 0.2 and 0.4 mg/L. TMP–SMX used as reference drug combination was tested at concentrations of 0.8/4, 1.6/8 and 3.2/16 mg/L. Preliminary experiments indicated that the final concentrations of methanol, acetone and dimethylsulphoxide (=0.1%) used in the dilution of drugs did not inhibit the growth of P. carinii. In experiments to test drug interactions, the final concentrations in the culture medium were 1, 10 and 20 mg/L for the lactoferrins, 1, 2 and 4 mg/L for clarithromycin and minocycline, and 0.1, 0.2 and 0.4 mg/L for pyrimethamine. Antibiotic-free plates were used as controls in the study. Experiments were performed in triplicate. P. carinii was added at a concentration of $10^2$–$10^5$ organisms per well. The monolayers were incubated at 37°C in 5% CO$_2$. After 72 h, 0.2 mL of supernatant containing nonadherent organisms was removed from each triplicate well and centrifuged (1800 g for 15 min); P. carinii vegetative and cystic forms (precystic forms and mature cysts) were quantified by counting P. carinii nuclei in duplicate Giemsa-stained 10 μL drops delivered on to 25 mm$^2$ squares on microscope slides. The A549 cells together with adherent P. carinii were removed from the plates by trituration following incubation in 1 mL of Ca$^{2+}$- and Mg$^{2+}$-free Hanks’ balanced salt solution containing 10 mM EDTA for 30 min at 37°C. The suspensions were centrifuged (1800 g for 15 min) and detached organisms were quantified by Giemsa staining, as described above.

The cytotoxicity of the lactoferrins was determined by the CellTitre 96 AQ cell proliferation assay (Promega Corp., Lyon, France). Control for each cytotoxicity assay included (i) uninfected cells incubated in DMEM; (ii) infected cells incubated in DMEM; and (iii) cells exposed to a freeze–thawed lysate containing $5 \times 10^3$ P. carinii equivalents in DMEM.

Analysis of results

The activity of each agent and combination was evaluated by counting viable parasites from plates with antimicrobial-supplemented medium and comparing it with the count from control plates without drugs (counts were total numbers of parasites in the cell layer and supernatant). The average number of P. carinii parasites per millilitre was calculated by counting 50 oil-immersion fields ($\times$1000 magnification) of each of three slides.

The 50% and 90% inhibitory concentrations (IC$_{50}$ and IC$_{90}$, respectively) of the drug were defined as the concentrations required to produce 50% and 90% reduction, respectively, in the mean cyst or trophozoite counts com-
pared with controls without drug after 72 h incubation in the presence of drugs.

The activity of each compound was also expressed by calculating the ratio of the cystic and trophic form numbers in cultures containing lactoferrin H or B at 20 mg/L, singly or in combination with other agents, to the number of cysts and trophozoites in control cultures after 72 h incubation.

Cytotoxicity was calculated from the following formula: ((mean optical density (OD) of uninfected cells – mean OD of infected cells)/mean OD of uninfected cells) × 100. Values of 0–5%, 6–25%, 26–50% and 51–100% were considered to indicate no toxicity, mild toxicity, moderate toxicity and severe toxicity, respectively, for A549 cells.

**Results**

In control plates without drugs, the number of *P. carinii* nuclei increased by at least three-fold over 3 days. In the absence of antibiotic, the mean number of parasites after 3 days was 49.5 (range 16–65) when calculated by counting 50 oil-immersion fields.

Lactoferrins H and B had similar effects (Table I). Lactoferrin B tested alone was more effective than lactoferrin H, with a higher activity against trophozoites than cysts. Lactoferrin B suppressed the growth of cysts and trophozoites by >60% at 20 mg/L (IC_{50} 20 mg/L). Lactoferrin H, at the same concentration, showed slightly less activity against cysts and trophozoites, producing >50% reduction in the mean cyst and nucleus counts (IC_{50} 20 mg/L). Neither lactoferrin B nor lactoferrin H achieved a 90% inhibitory effect at the concentrations tested, so their IC_{50} is >20 mg/L.

The results with combinations of agents are shown in Tables II and III. The agents used in combination were superior to single-agent activity in reducing the cyst and trophozoite counts. The activity of lactoferrins was improved when these compounds were combined with all other compounds at the highest concentrations tested. Lactoferrin B 20 mg/L combined with clarithromycin 4 mg/L showed the highest anti-*P. carinii* activity, with a decrease

**Table I.** Anti-pneumocystis activity of lactoferrin B and lactoferrin H alone or in combination with other antimicrobial agents

<table>
<thead>
<tr>
<th>Antimicrobial agent(s)*</th>
<th>Drug concentrations (mg/L)</th>
<th>Peak ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/L</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Lactoferrin B alone</td>
<td>0.39</td>
<td>0.37</td>
</tr>
<tr>
<td>Lactoferrin B + clarithromycin</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td>Lactoferrin B + pyrimethamine</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>Lactoferrin B + minocycline</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td>Lactoferrin H alone</td>
<td>0.42</td>
<td>0.41</td>
</tr>
<tr>
<td>Lactoferrin H + clarithromycin</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Lactoferrin H + pyrimethamine</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>Lactoferrin H + minocycline</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>Trimethoprim–sulphamethoxazole</td>
<td>0.08</td>
<td>0.06</td>
</tr>
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</table>

Values of 0–5%, 6–25%, 26–50% and 51–100% were considered to indicate no toxicity, mild toxicity, moderate toxicity and severe toxicity, respectively, for A549 cells.

**Table II.** Inhibitory effects of lactoferrin B in combination with other agents

<table>
<thead>
<tr>
<th>Drug/concentration (mg/L)</th>
<th>0 mg/L</th>
<th>1 mg/L</th>
<th>10 mg/L</th>
<th>20 mg/L</th>
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<tr>
<td></td>
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<td>Minocycline</td>
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<td></td>
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<td>0</td>
<td>0</td>
<td>8.4</td>
<td>9.5</td>
</tr>
<tr>
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<td>3.3</td>
<td>4.1</td>
<td>14.6</td>
<td>16.8</td>
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<td>4</td>
<td>9.5</td>
<td>11.4</td>
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<tr>
<td>Pyrimethamine</td>
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<td>10.3</td>
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<tr>
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<td>8.9</td>
<td>20.8</td>
<td>22.3</td>
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</table>
of 94.4% and 98.1% in cyst and trophozoite counts, respectively. In contrast, lactoferrins in combination with pyrimethamine and minocycline did not reduce trophozoite and cyst numbers by >90%, except the combination of lactoferrin B with pyrimethamine or minocycline against trophozoites. The only combination that achieved a 90% inhibitory effect for trophozoites or cysts was the combination of clarithromycin with either of the two lactoferrins.

TMP–SMX suppressed the growth of cysts and trophozoites by 60% at 0.8/4 mg/L and 90% at 3.2/16 mg/L and 1.6/8 mg/L (Table I).

Ratios of the peak number of viable organisms in drug-treated cultures to the peak number in control cultures showed that lactoferrin B 20 mg/L showed the highest activity against both cysts and trophozoites (peak ratio 0.39 and 0.37, respectively). Lactoferrin H tested alone at 20 mg/L showed similar activity (cyst ratio, 0.42; trophozoite ratio, 0.41). Lactoferrin B 20 mg/L in combination with clarithromycin 4 mg/L had in vitro anti-P. carinii activity (cyst ratio, 0.06; trophozoite ratio, 0.02) comparable to that of TMP–SMX (cyst ratio, 0.06; trophozoite ratio, 0.08). These results are summarized in Table I.

The cytotoxicity of lactoferrins alone (~7.3% to 12.2% cytotoxicity) and in all combinations (~5.8% to 8.3% cytotoxicity) was very low.

**Discussion**

*P. carinii* is a common pathogen that frequently causes pulmonary infection in immunocompromised individuals. In this study, we focused our attention on lactoferrin, since it has been demonstrated that this milk protein not only can inhibit the growth of several bacteria and *Candida* spp. but also has important activity against several viruses, such as HIV and herpes simplex virus. Lactoferrins are iron-binding proteins found in mucosal secretions and in the specific granules of polymorphonuclear leukocytes. A variety of functions have been ascribed to this protein although its physiological role remains to be defined fully. It appears to contribute to antimicrobial host defence. It has direct effects on pathogenic microorganisms, including bacteriostasis and the induction of microbial iron uptake systems. It has been suggested that the antimicrobial activity of lactoferrin arises from its sequestration of environmental iron, which would cause nutritional deprivation in susceptible organisms. However, a number of other potential mechanisms by which lactoferrin inhibits the growth of several microorganisms have been suggested, including structural changes in the microbial cell wall, complete loss of membrane potential and integrity, indirect effects on enzyme activation, an increased generation of metabolic by-products of aerobic metabolism, iron deprivation and combinations of these factors.

The antimicrobial activities of lactoferrins make it a promising compound for combination therapy. The drugs tested in combination with lactoferrin in this study have distinct modes of action. Pyrimethamine is a specific inhibitor of dihydrofolate reductase in many microorganisms; this enzyme is involved in folate synthesis. Pyrimethamine has only a static effect on parasite growth. The antimicrobial activity of macrolides and tetracyclines results from their ability to inhibit protein synthesis by binding to the
transpeptidation site of the larger ribosomal subunit. Studies of pharmacokinetic interaction of clarithromycin and other antimicrobial agents have shown that this macrolide, by inhibiting the cytochrome P-450 pathway, may reduce the metabolism of these agents, resulting in increased serum and tissue concentrations. In our experiments, lactoferrin had good activity against *P. carinii*, and this activity was enhanced when lactoferrin was combined with other drugs. The most active combination tested was lactoferrin plus clarithromycin. This result is in line with other studies that reported anti-*P. carinii* activity when lactoferrins were combined with clarithromycin, pyrimethamine or minocycline. This interaction mechanism appears to be complex. As mentioned above, lactoferrin has multiple mechanisms of antimicrobial action, while clarithromycin and minocycline inhibit protein synthesis and pyrimethamine indirectly inhibits folate synthesis. The additive effect of these compounds may result from the cumulative inhibitory effect on different, essential metabolic pathways.

The cytotoxicity assay indicates that these combinations are safe. Our results showed that the combinations tested were active in inhibiting *P. carinii* at concentrations which appeared to be non-toxic for the human cell monolayer.

In conclusion, our results lend experimental support to the use of iron-chelating agents such as lactoferrin, alone or in combination with macrolides, tetracyclines or dihydrofolate reductase inhibitors in *P. carinii* pneumonia.

References


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