Inhibitory effects of human neutrophil granules and oxygen radicals on adherence of Candida albicans

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Summary. The adherence of Candida albicans to dacron fibre microcolumns was significantly suppressed after interaction with human neutrophils. The adherence-inhibiting properties of neutrophils were shown to reside in their cytoplasmic granules and granular enzymes. Oxygen-derived free radicals produced by the respiratory burst may also be responsible, as shown by experiments in which oxygen radicals were generated by the cell-free hypoxanthine-xanthine oxidase system. Dose-response studies with H₂O₂ and β-glucoronidase demonstrated that lower concentrations of these agents inhibited adherence without affecting viability of C. albicans. These results suggest that interference with adherence mechanisms may be an effective means of host defence by neutrophils against the colonisation of mucosal surfaces by C. albicans.

Introduction

Candida albicans is an opportunist microbial pathogen that causes a spectrum of infections from localised, self-limiting lesions of skin or mucous membranes to fatal disseminated disease. Although its invasive mechanisms are not well understood, the capacity to adhere to host cells, prosthetic devices and catheters represents a crucial early event in the pathogenesis of infection (Sobel et al., 1981; Lee and King, 1983; Shepherd et al., 1985; Rotrosen et al., 1986). The central role of neutrophils in defence against candida infection has been documented by in-vitro studies and clinical observations (Bodey, 1966; Kirkpatrick et al., 1971; Lehrer and Cline, 1971; Lehrer et al., 1975). This defence is mediated by various candidicidal proteins and enzymes located in cytoplasmic granules, and oxygen radicals generated by the respiratory burst (Lehrer, 1972; Diamond et al., 1980; Gantz et al., 1985; Selstead et al., 1985). Interference with adherence mechanisms of C. albicans has not been investigated previously as a possible target for immunological attack, although it has been postulated as a possible mode of action of antifungal drugs (Vuddhakul et al., 1988). Therefore it was decided to examine the ability of neutrophils and their microbial constituents to interfere with the adherence of candida.

Materials and methods

C. albicans

A recent clinical isolate of C. albicans from the blood of a patient with an infected intravenous catheter, and designated P12282, was kindly provided by Dr M. Tilse, Department of Microbiology, Mater Public Hospital, and maintained in culture as previously described with minor modifications (Thong and Ferrante, 1978; Ferrante and Thong, 1979). The stock culture was maintained at 25°C in Sabouraud Dextrose Agar (SDA) slopes covered with paraffin. A loopful of stock culture was subcultured on SDA for 18 h at 37°C, and then inoculated into SDA broth and incubated at 25°C for 18 h. A sample was removed, washed twice and resuspended in Dulbecco's phosphate buffered saline with Ca+++, and Mg+++ glucose 1% (DPBS, Flow Laboratories, Sydney, Australia), phosphate buffered saline (PBS), or normal (physiological) saline for the experiments.

Neutrophil isolation

Neutrophils were prepared from the heparinised blood of healthy volunteers by centrifugation on Mono-Poly Resolving Medium (Flow Laboratories) at 1000 g for 30 min. Neutrophils of >97% purity were recovered from the second band at the interface (Ferrante and Thong, 1980). The cells were washed twice and resuspended in either DPBS, PBS or normal saline and adjusted to the required concentration by counting in a Neubaur haemocytometer.

Received 26 Aug. 1988; revised version accepted 17 Nov. 1988.
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Neutrophil granules

Neutrophils (5 x 10⁷ cells) in ice-cold relaxation buffer were disrupted by three bursts, each of 2 s, with a Branson ultrasonicator (West et al., 1974). Nuclei and nongranular debris were removed by successive filtration through 5-μm and 2-μm membrane filters (Millipore). Granules were separated on discontinuous Percoll gradients (Borregaard et al., 1983) consisting of 4 ml of Percoll, density 1.120 g/ml, layered under 4 ml of Percoll, density 1.050 g/ml. Approximately 1.5 ml of neutrophil extract was applied to the top and the gradient was centrifuged at 4°C and 17 000 g for 15 min. Three distinct bands were formed—azurophil granules, specific granules and plasma membrane. The purity of these bands were ascertained by assay for β-glucuronidase activity, which is confined to azurophil granules (Seow et al., 1988). Percoll from each granule band was removed by centrifugation at 23 000 g for 2 h, and the granules were dispersed in PBS by six bursts, each of 5 s, with a Branson ultrasonicator.

Oxygen metabolites

Oxygen radicals were generated in vitro by the hypoxanthine-xanthine oxidase cell-free system as previously described (Seow et al., 1988). Hypoxanthine (HX, Sigma, St Louis MO, USA) was dissolved in 0.1 m PBS. Xanthine oxidase (XO, Boehringer, Sydney, Australia) was dissolved in 2 m ammonium sulphate. Experiments were performed with a mixture of 0.1 mM hypoxanthine and 4 mU of xanthine oxidase in 1 ml-volumes containing C. albicans at a final concentration of 5 x 10⁶ yeast cells/ml. After incubation for 1 h at 37°C, the cells were washed twice and adherence measured by the method described below.

Adherence micro-assay

This assay was performed as previously described (Seow et al., 1987c; Vuddhakul et al., 1988). Briefly, dacron fibre microcolumns were prepared by carefully weighing 10-μg lots of dacron fibre (Olympic General Products, Queensland, Australia) and placing them into 100-μl disposable pipette tips (Stockwell Scientific, CA, USA) so as to occupy the middle 1.5 cm of the 5 cm length of the pipette tip. Each microcolumn so prepared can accommodate 100 μl of the C. albicans suspension.

After pre-incubation of C. albicans suspensions with neutrophils, granule constituents, oxygen radicals or specific enzymes, 10-μl volumes of C. albicans, at a concentration of 5 x 10⁶ cells/ml, were pipetted into the dacron fibre microcolumns and kept at room temperature in high humidity for 30 min. The microcolumns were then placed on a specially constructed apparatus which applied a suction pressure of approximately 250 mbar for 1 min as previously described (Thong and Currell, 1983). The effluents were collected in disposable test tubes and chilled in ice before quantitation of yeast cells with a Neubauer haemocytometer. Experiments were performed in triplicate and the results were calculated as follows:

\[
\text{Percentage adherence} = \frac{100 - (C_e/Co \times 100)}{C_e/Co \times 100}
\]

where Co is the original concentration of C. albicans applied, and Ce is the concentration recovered in the effluent.

Fungal viability assay

Viability of C. albicans after exposure to H₂O₂ or β-glucuronidase was assessed by colony counting (Hughes et al., 1987). Samples were removed and serially diluted in sterile saline, and 0.05-ml volumes were spread on SDA plates. The samples were also checked by microscopy to exclude the possibility of aggregation of yeast cells which may contribute to error in this type of assay. Cultures were placed in incubators at 37°C for 24-48 h, after which the number of viable colonies was counted. Experiments were performed in triplicate and results were expressed as mean (SD)% adherence.

Statistical analysis

All experimental results were analysed by the one-way Analysis of Variance (ANOVA). Where appropriate, further statistical analysis was performed by the multiple comparisons procedure devised by Newman-Keuls (Miller, 1981; Milliken and Johnson, 1984).

Results

Effect of neutrophils on candida adherence

Initially the ability of human neutrophils to inhibit the adherence of C. albicans was investigated. C. albicans suspensions, at a concentration of 1 x 10⁷ cells/ml, were divided into three lots. One lot was mixed with an equal volume of unstimulated neutrophils (1 x 10⁷ cells/ml), another with neutrophils which had been stimulated for 30 min with phorbol myristate acetate (PMA, Sigma 0.1 μg/ml) (Seow et al., 1987b), and the third lot served as control to which PBS only was added. After incubation at 37°C for 30 min, the neutrophils were lysed with sodium desoxycholate 2-5% (Diamond et al., 1978) and washed twice in PBS, and the adherence of candida was determined. Preliminary experiments showed that this concentration of sodium desoxycholate has no effect on yeast adherence. The results (table I) show that unstimulated neutrophils significantly suppressed the adherence of candida (p<0.05). Neutrophils stimulated by PMA caused an even greater suppression of yeast adherence (p<0.01) compared to control (saline) preparations.
performed in triplicate with neutrophil granules from separate donors. F test), with greater inhibition by azurophil granules (p < 0.01, Newman-Keuls).

The results (table 1) show that, while both types of neutrophil granules were isolated and examined for their capacity to inhibit the adherence of Candida albicans. Suspensions of C. albicans were incubated with equal volumes of either specific or azurophil granules for 60 min, washed twice in PBS, and adherence was then measured. Each experiment was performed in triplicate with neutrophils from separate individuals.

Statistical analysis by one-way ANOVA shows significant inhibition of adherence by human neutrophils (p < 0.01). The multiple comparisons test (Newman-Keuls) showed that PMA-stimulated neutrophils have greater inhibitory effects than unstimulated neutrophils (p < 0.05).

Effect of specific and azurophil granules

The multiple comparisons test (Newman-Keuls) showed that the hyphae of C. albicans were 5 x 10⁶/ml (ratio 1:1). Incubation time was 1 h at 37°C. PMA-stimulated neutrophils were pre-treated with PMA 0.1 µg/ml for 30 min before interaction with C. albicans. Each experiment was performed in triplicate with neutrophils from separate individuals.

Statistical analysis by one-way ANOVA shows significant inhibition of adherence by human neutrophils (p < 0.01, F test). The multiple comparisons test (Newman-Keuls) showed that PMA-stimulated neutrophils have greater inhibitory effects than unstimulated neutrophils (p < 0.05).

The final concentrations of neutrophils and C. albicans were 5 x 10⁶/ml (ratio 1:1). Incubation time was 1 h at 37°C. PMA-stimulated neutrophils were pre-treated with PMA 0.1 µg/ml for 30 min before interaction with C. albicans. Each experiment was performed in triplicate with neutrophils from separate individuals.

Statistical analysis by one-way ANOVA shows significant inhibition of adherence by human neutrophils (p < 0.01, F test). The multiple comparisons test (Newman-Keuls) showed that PMA-stimulated neutrophils have greater inhibitory effects than unstimulated neutrophils (p < 0.05).

Effect of oxygen metabolites

The effects of oxygen-derived free radicals on yeast adherence are shown in table IV. Incubation of C. albicans with a mixture of hypoxanthine (HX) and xanthine oxidase (XO) resulted in a significant reduction in candida adherence (p < 0.01). No inhibition of adherence was observed for C. albicans treated with either HX or XO alone. Known oxygen-radical scavengers such as superoxide dismutase (SOD, Sigma), catalase (Boehringer), di-methyl-sulfoxide (DMSO, Boehringer) and tetrandrine (Seow et al., 1988) were able to reverse the inhibition caused by HX and XO (tables V and VI). Heat-inactivation of SOD and catalase rendered them ineffective in this regard (tables V and VI).

Adherence-inhibition and fungal viability

C. albicans was subjected to varying concentrations of either H₂O₂ or β-glucuronidase to determine whether inhibition of yeast adherence occurs without impairment of fungal viability. The results (table VII) show dose dependent inhibition of both yeast adherence and viability. However, lower concentrations of either H₂O₂ or β-glucuronidase caused significant inhibition of adherence without affecting viability.

Discussion

The fungicidal properties of neutrophil leucocytes are known to reside in their cytoplasmic granules and in oxygen-derived free radicals generated by the respiratory burst. It is also known that the hyphae of C. albicans have the capacity to stimulate the respiratory burst and degranulation by neutrophils without the intervention of serum opsonins (Diamond and Krzesicki, 1978; Diamond et al., 1978; Lyman et al., 1987). This is particularly relevant to colonisation of the mucosa by C. albicans because serum opsonins are only present in minute quantities.
Table III. Effect of enzymes found in neutrophil granules on the adherence of C. albicans

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Untreated C. albicans</th>
<th>Lysozyme</th>
<th>β-glucuronidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>native</td>
<td>heated</td>
<td>native</td>
</tr>
<tr>
<td>1</td>
<td>48.1(3.1)</td>
<td>32.0(3.4)</td>
<td>45.7(4.0)</td>
</tr>
<tr>
<td>2</td>
<td>61.8(4.2)</td>
<td>40.8(3.8)</td>
<td>59.8(4.1)</td>
</tr>
<tr>
<td>3</td>
<td>55.1(3.2)</td>
<td>39.0(3.5)</td>
<td>50.4(3.3)</td>
</tr>
</tbody>
</table>

C. albicans was pre-treated with native or heat-treated (65°C for 30 min) enzymes for 1 h at 37°C before assay of yeast adherence. Each of the experiments was performed in triplicate on separate occasions.

Statistical analysis by one-way ANOVA and Newman-Keuls showed significant inhibition of yeast adherence by native but not heat-inactivated enzymes (p < 0.01).

C. albicans was incubated with hypoxanthine (HX), xanthine oxidase (XO) or a combination of HX and XO for 1 h at 37°C before assay of adherence. Each experiment was performed in triplicate on separate occasions.

One-way ANOVA and Newman-Keuls analysis showed significant inhibition by the combination of HX and XO (p < 0.01).

Table IV. Effect of oxygen-derived free-radicals on the adherence of C. albicans

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Untreated C. albicans</th>
<th>HX</th>
<th>XO</th>
<th>HX+XO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59.3(4.2)</td>
<td>50.3(7.4)</td>
<td>53.9(4.2)</td>
<td>24.4(12.4)</td>
</tr>
<tr>
<td>2</td>
<td>48.5(5.4)</td>
<td>49.2(8.2)</td>
<td>30.9(1.2)</td>
<td>30.7(4.5)</td>
</tr>
<tr>
<td>3</td>
<td>43.2(4.6)</td>
<td>45.8(2.7)</td>
<td>42.0(2.3)</td>
<td>22.0(5.5)</td>
</tr>
</tbody>
</table>

C. albicans was incubated with the combination of hypoxanthine (HX) and xanthine oxidase (XO) or a combination of HX and XO for 1 h at 37°C before assay of adherence. The reaction mixture also contained superoxide dismutase (SOD) or catalase, in the native (n) or the heat-inactivated (i, 65°C for 30 min) forms. Experiments were performed in triplicate on separate occasions.

One-way ANOVA and Newman-Keuls statistical analysis showed reversal of adherence inhibition in the presence of native SOD and catalase (p < 0.01), but not with the heat-inactivated forms.

Table V. Inhibition of C. albicans adherence by oxygen radicals and reversal by natural scavengers

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Untreated C. albicans</th>
<th>HX + XO</th>
<th>HX + XO SOD(n)</th>
<th>HX + XO SOD (i)</th>
<th>HX + XO catalase (n)</th>
<th>HX + XO catalase (i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66.8(9.3)</td>
<td>34.2(5.3)</td>
<td>53.9(6.5)</td>
<td>24.5(5.9)</td>
<td>61.5(4.9)</td>
<td>28.4(6.5)</td>
</tr>
<tr>
<td>2</td>
<td>60.9(6.2)</td>
<td>23.2(3.9)</td>
<td>55.7(8.9)</td>
<td>27.0(3.6)</td>
<td>52.5(5.7)</td>
<td>35.7(5.1)</td>
</tr>
<tr>
<td>3</td>
<td>49.7(5.1)</td>
<td>31.4(6.9)</td>
<td>45.2(2.3)</td>
<td>35.9(4.9)</td>
<td>47.3(5.5)</td>
<td>29.4(7.3)</td>
</tr>
</tbody>
</table>

C. albicans was incubated with the combination of hypoxanthine (HX) and xanthine oxidase (XO) for 1 h at 37°C before assay of adherence. The reaction mixture also contained superoxide dismutase (SOD) or catalase, in the native (n) or the heat-inactivated (i, 65°C for 30 min) forms. Experiments were performed in triplicate on separate occasions.

One-way ANOVA and Newman-Keuls statistical analysis showed reversal of adherence inhibition in the presence of native SOD and catalase (p < 0.01), but not with the heat-inactivated forms.
peptides and some enzymes (Lehrer et al., 1975; Lehrer and Ladra, 1977; Gantz et al., 1985). It was decided to use lysozyme, found in both specific and azurophil granules, and β-glucuronidase, present in azurophil granules only, because their mechanisms of action are well defined. Thus lysozyme has been noted to cause an increase in permeability of the fungal cell membrane (Collins and Pappagianis, 1974a,b), whereas β-glucuronidase is believed to cause degradation of oligosaccharides in the fungal cell wall (Podhradsky et al., 1982). The experiments also showed that oxygen radicals produced by the hypoxanthine-xanthine oxidase reaction are also capable of suppressing the adherence of candida and that this suppression can be reversed by a number of antioxidants and oxygen radical scavengers. With known concentrations of enzyme or oxygen radicals, we showed, in subsequent experiments, that the concentrations required for inhibition of adherence were much less than those needed for killing C. albicans.

Since dacron fibres, a synthetic material, was used to measure candida adherence in these studies, the clinical relevance of our data to candidal colonisation of dentures and indwelling catheters may be inferred. Whether or not there is relevance to candida infection of mucosal surfaces is unclear, although there is some evidence to suggest that the same "adhesin" mechanism of C. albicans may be involved, and that there may be a correlation between adherence to plastic materials and to mucosal surfaces (Samaranayake and MacFarlane, 1980; Samaranayake et al., 1980; Minagi et al., 1985; Rotrosen et al., 1986).

These results suggest that the adherence mechanism of C. albicans may be a feasible target for immunological attack by neutrophils, and since less energy and materials are needed for adherence inhibition than for fungicidal activity, this may be an effective method of host defence against the colonisation of mucosal surfaces by C. albicans.

### Table VI. Inhibition of C. albicans adherence by oxygen radicals and reversal by anti-oxidants

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Untreated C. albicans</th>
<th>HX + XO</th>
<th>HX + XO</th>
<th>HX + XO</th>
<th>DMSO</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>53.1(8.9)</td>
<td>23.9(3-0)</td>
<td>42.8(4-4)</td>
<td>38.2(6-5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>60.1(5-1)</td>
<td>38.7(5-1)</td>
<td>54.9(6-7)</td>
<td>53.9(7-4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>57.1(7-6)</td>
<td>36.5(3-5)</td>
<td>60.4(4-8)</td>
<td>52.5(6-8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. albicans was incubated with a combination of hypoxanthine (HX) and xanthine oxidase (XO) in the presence of dimethylsulfoxide (DMSO) or tetrandrine (TT), for 1 h at 37°C before assay of adherence. Experiments were performed in triplicate on separate occasions.

Statistical analysis by one-way ANOVA and Newman-Keuls statistical analysis showed significant inhibition of yeast adherence at lower concentrations of H2O2 or β-glucuronidase compared to yeast viability.

### Table VII. Effect of H2O2 and β-glucuronidase on C. albicans adherence and viability

<table>
<thead>
<tr>
<th>H2O2 concentration (μM)</th>
<th>Experiment no. 1</th>
<th>Experiment no. 2</th>
<th>Experiment no. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>adherence</td>
<td>viability</td>
<td>adherence</td>
</tr>
<tr>
<td>0</td>
<td>52.8(8.8)</td>
<td>6.5(1.5)</td>
<td>73.5(4.3)</td>
</tr>
<tr>
<td>1</td>
<td>48.7(5.5)</td>
<td>5.7(0.9)</td>
<td>72.1(2.6)</td>
</tr>
<tr>
<td>10</td>
<td>40.4(6.1)</td>
<td>5.2(1.7)</td>
<td>65.6(4.2)</td>
</tr>
<tr>
<td>100</td>
<td>17.0(6.0)</td>
<td>3.9(1.4)</td>
<td>40.3(3.7)</td>
</tr>
<tr>
<td>500</td>
<td>3.3(3.6)</td>
<td>1.4(0.5)</td>
<td>11.6(3.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>β-glucuronidase concentration (U/ml)</th>
<th>Experiment no. 1</th>
<th>Experiment no. 2</th>
<th>Experiment no. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60.1(4.8)</td>
<td>5.4(0.8)</td>
<td>65.6(5.5)</td>
</tr>
<tr>
<td>5</td>
<td>57.9(4.3)</td>
<td>5.0(1.1)</td>
<td>61.7(4.7)</td>
</tr>
<tr>
<td>10</td>
<td>30.0(1.6)</td>
<td>4.2(0.5)</td>
<td>44.9(5.7)</td>
</tr>
<tr>
<td>20</td>
<td>19.4(5.6)</td>
<td>4.1(0.7)</td>
<td>25.0(4.1)</td>
</tr>
<tr>
<td>40</td>
<td>9.1(4.9)</td>
<td>3.0(0.3)</td>
<td>13.9(4.6)</td>
</tr>
</tbody>
</table>

C. albicans was incubated with H2O2 or β-glucuronidase for 1 h at 37°C before the simultaneous assays for adherence and viability. Experiments were performed in triplicate and results expressed as mean percentage (SD) adherence or viability. Each experiment was performed on separate occasions.

One-way ANOVA and Newman-Keuls statistical analysis showed significant inhibition of yeast adherence at lower concentrations of H2O2 or β-glucuronidase compared to yeast viability.
this regard, there is increasing evidence to suggest that neutrophils play a significant role in defence at mucosal surfaces (Attstrom and Egelberg, 1970; Scully and Challacombe, 1979; Seow et al., 1987a, b; Tsuda et al., 1983; Weiss et al., 1983).

The nature of adherence mechanisms in \( C. \) albicans is not as well understood as that of bacteria (Beachy, 1981) but may involve mannoprotein adhesins (McCourtie and Douglas, 1981). These cell wall structures appear to be as susceptible to interactions mediating the attachment of bacteria to mucosal surfaces (Beachy, 1981) but may involve mannoprotein adhesins (McCourtie and Douglas, 1981). These cell wall structures appear to be as susceptible to interactions mediating the attachment of bacteria to mucosal surfaces (Beachy, 1981) but may involve mannoprotein adhesins (McCourtie and Douglas, 1981). These cell wall structures appear to be as susceptible to interactions mediating the attachment of bacteria to mucosal surfaces (Beachy, 1981) but may involve mannoprotein adhesins (McCourtie and Douglas, 1981). These cell wall structures appear to be as susceptible to interactions mediating the attachment of bacteria to mucosal surfaces.

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