Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an in vitro model of oral candidosis

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The immune response and the anticandidal activity of keratinocytes and polymorphonuclear leukocytes (PMNs) play a key role in host defence against localized Candida albicans infection. An established model of oral candidosis based on reconstituted human oral epithelium (RHE) was supplemented with PMNs to study the effect of these immune cells during experimental oral candidosis. Infection of RHE with C. albicans induced a strong expression of the chemokine interleukin-8 (IL-8) and the cytokine granulocyte-macrophages colony-stimulating factor (GM-CSF), and a moderate stimulation of interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) by keratinocytes. This immune response was associated with chemoattraction of PMNs to the site of infection, whereas uninfected RHE failed to induce cytokine expression or to attract PMNs. Growth of the pathogen and tissue damage of C. albicans-infected RHE were significantly reduced when PMNs were applied to the apical epithelial surface or when PMNs migrated through a perforated basal polycarbonate filter of the model. Notably, protection against epithelial tissue damage was also observed when PMNs were placed on the basal side of non-perforated filters, which prevented PMN migration into the RHE. Addition of PMNs enhanced a Th1-type immune response (IFN-γ, TNF-α), down-regulated the expression of the Th2-type cytokine interleukin-10 (IL-10), and was associated with protection against Candida-induced tissue damage. This PMN-supplemented model of oral candidosis mimics the in vivo situation, and provides a promising tool for studying the immunological interactions between keratinocytes and C. albicans, as well as the influence of PMNs on C. albicans pathogenesis.

INTRODUCTION
Keratinocytes and neutrophils, eosinophils or basophils (polymorphonuclear leukocytes, PMNs) are the first line of host defence against mucosal Candida albicans infections (Challacombe, 1994; Eversole et al., 1997). An important step in the inflammatory response to mycotic infection leading to the recovery from infection is a Th1-type immune response mediated by CD4+ T-cells (Fidel, 2002a). Furthermore, transepithelial migration of PMNs represents the histological hallmark of oral candidal lesions and is believed to play a crucial role in the clearance of infection (Fidel, 2002a). This is in line with the fact that patients suffering from defects in neutrophil function or neutropenic patients are more susceptible to oral colonization and infection with C. albicans (Epstein et al., 2003; Martino et al., 1989; Myoken et al., 2004; Walsh et al., 1996; Wolff, 1972). In vitro candidacidal activity has been observed for both keratinocytes and PMNs (Fidel, 2002b; Romani, 1999). In addition, specific monoclonal antibodies have been used to deplete PMNs in the systemic circulation of mice in order to study their influence on the pathogenesis of C. albicans infections and to mimic the in vivo situation in neutropenic patients suffering from vaginal (Black et al., 2002a).
1998; Fidel et al., 1999) and oral candidosis (Farah et al., 2001). However, there are contradictory data about the role of PMNs in resolving murine oral candidosis in different mouse strains (Farah et al., 2001). In a recent study, we characterized the immune response of keratinocytes during interaction with C. albicans using reconstituted human oral epithelium (RHE) (Schaller et al., 2002). To establish a more physiological infection model, we supplemented the RHE with PMNs and studied their impact on the pathogenesis of experimental oral infections and the cytokine response.

METHODS

Culture media and growth conditions. For the infection of the reconstituted oral epithelium (RHE), inocula were prepared by culturing the clinical C. albicans wild-type strain SC5314 (Gillum et al., 1984) for 24 h at 37°C on Sabouraud dextrose agar (Difco). Cells were washed three times in 0-9% NaCl and 2 x 10^6 cells were then suspended in 10 ml YPG medium (Difco). The suspension was cultured for 16 h at 25°C with orbital shaking. A suspension of 4 x 10^6 cells from this culture was incubated with shaking in fresh medium for 24 h at 37°C. After washing three times with PBS, the final inoculum was adjusted to the desired density with PBS.

Isolation of polymorphonuclear cells (PMNs). PMNs obtained from healthy human volunteers were isolated from heparinized whole blood using Histopaque-1119 in combination with Histopaque-1077 (Sigma), according to the manufacturer’s protocol. The cells recovered from the interface were washed three times and suspended at a concentration of 2 x 10^8 ml^(-1) in RPMI 1640 medium (Sigma) in the presence and absence of 10% fetal calf serum (FCS). Residual erythrocytes were removed by hypotonic lysis. Giemsa staining and light microscopy were used to ensure that a pure population of PMNs (>90% purity) with typical morphology had been isolated. The cells were vital-stained using the trypan blue dye-exclusion method. Numbers of vital and non-vital leukocytes per sample were assessed using a Neubauer chamber. The viability was ≥95% in all experiments.

Reconstituted human oral epithelium and model of oral candidosis. The reconstituted human epithelium (RHE) for the in vitro model of oral candidosis was supplied by Skinethic Laboratory (Nice, France). It was obtained by culturing transformed human keratinocytes of the cell line TR146 derived from a carcinoma of the oral epithelium (Rupniak et al., 1985). Keratinocytes were incubated in serum-free conditions in a defined medium based on MCDB-153 medium (Clonetics), containing 5 μg insulin ml^(-1), on a 0.5 cm² microporous polycarbonate filter for 7 days at the air–liquid interface. TR146 cells form a three-dimensional epithelial tissue resembling human oral mucosa in vivo (Fig. 1a). The in vitro model and all culture media were prepared without antibiotics and antimycotics. Five infection experiments were performed for the C. albicans strain SC5314. RHE was infected with 2 x 10^9 Candida cells in 50 μl PBS for 12 and 24 h (Fig. 1b). Non-infected controls contained 50 μl PBS alone.

Supplementation with PMNs and transepithelial migration assay. The model of oral candidosis was supplemented with PMNs in three different ways (Fig. 1c–e). (1) PMNs (10^7 cells in 50 μl RPMI 1640 medium, with and without 10% FCS) were directly added to the apical epithelial layers of the model 6 or 12 h after infection with C. albicans SC5314 (Fig. 1c). (2) Other samples of the preinfected model were inverted after 6 or 12 h, and PMNs were added to the basal side of the polycarbonate filter. In these samples, PMNs were not able to migrate through the microporous filter (Fig. 1d). (3) In a subset of these samples, the polycarbonate filter was perforated with a thin needle before addition of the PMNs to enable transepithelial migration. All samples were incubated for a further 6 or 12 h after addition of the immune cells. Culture medium was applied to the basal side of the filter every 60 min to feed the cells. Histological sections and LDH analysis of the supplemented samples were compared to that of the non-supplemented inverted model of oral candidosis (f).
and uninfected RHE after addition of RPMI 1640/10% FCS medium without PMNs, and uninfected RHE supplemented with PMN cells in the same way as described above. Culture medium was added to the basal side of the inverted samples every 60 min to feed the keratinocytes/PMNs with nutrients.

**Assay of lactate dehydrogenase activity.** The release of lactate dehydrogenase (LDH) from epithelial cells into the surrounding medium was monitored as a measure of epithelial cell damage. LDH release in the maintenance media of the cultures from uninfected and infected epithelial cells was measured at 12 and 24 h. LDH activity was analysed spectrophotometrically by measuring the rate of NADH disappearance at 340 nm during the LDH-catalysed conversion of pyruvate to lactate, according to the Wróblewski–La Due method (Wróblewski & John, 1955). The LDH activity is given as U l⁻¹ at 37 °C.

**Killing assay.** A killing assay was used to study the inhibitory effect of PMNs on *C. albicans* during RHE infection. It was performed by plating the infected samples 12 and 24 h after incubation, with and without PMN, on Sabouraud dextrose agar. Before plating, 2 ml PBS was added to the samples, and samples were thoroughly vortexed for 10 min. Furthermore, the solution was vigorously agitated by using a Pasteur pipette to separate big clumps of fungal cells into single cells, and then diluted 1:1000 and 1:10 000 in PBS. The presence of single cells was confirmed by light microscopy. Yeast cell viability was determined by assessment of the colony forming units produced after incubation for 24 h at 37 °C on Sabouraud dextrose agar.

**Light microscopy.** Light-microscopy studies were performed to evaluate histological changes during infection. Part of each specimen was fixed, postfixed and embedded in glycol methacrylate, and cut using an ultramicrotome (Ultracut). Semi-thin sections (1 μm) were studied with a light microscope after staining with 1% toluidine blue and 1% pyronine G (Merck). The histological changes of the mucosa were evaluated on the basis of 50 sections from five different sites for each infected epithelium.

**RNA isolation and cDNA-synthesis by reverse transcriptase.** For the detection of mRNA, samples were rapidly removed and shock-frozen in liquid nitrogen. Total RNA from shock-frozen samples was isolated using RNAPure (Peqlab), according to the manufacturer’s instructions. For assessing RNA concentration and purity, UV spectroscopy was used (Bio Photometer, Eppendorf). The absorbance of a diluted RNA sample was measured at 260 and 280 nm. cDNA synthesis was performed using Superscript II reverse transcriptase ( Gibco), following the manufacturer’s instructions.

**Quantitative RT-PCR (QRT-PCR).** For cytokine expression, 20 ng of cDNA were amplified ‘real-time’ in a LightCycler (Roche), using a First-Strand DNA Master SYBR Green I kit (Roche) at 3 mM Mg²⁺ final concentration, and analysed with LightCycler Software 3.3. Annealing temperature and elongation time were optimized for each primer pair. The sequences of the primer pairs used were published recently (Schaller et al., 2002). Amplified DNA for each primer pair was serially diluted (six logs) and used to generate standard curves. Absolute quantification for these cDNAs was achieved with the LightCycler software.

**Quantification of cytokine secretion by epithelial cells stimulated with *C. albicans*.** Epithelial tissues (with or without PMNs; see above) were infected with PBS-washed *C. albicans* S5314 or treated with PBS only. After 12 h and 24 h, samples of the maintenance medium surrounding the infected and uninfected epithelial tissues were collected and centrifuged. The amount of interleukin-1β (IL-1β), IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) secreted into the supernatant was determined by fluorescence-activated cell sorting (FACS) analysis. Flow cytometric data were acquired on a Becton Dickinson FACScan using the Human Inflammation Kit (Becton Dickinson). Data were analysed using Cellquest software (Becton Dickinson).

**RESULTS**

**Protective effect and transepithelial migration of PMNs during infection of oral epithelium with *C. albicans*.**

After 12 and 24 h infection with *C. albicans*, the RHE showed signs of tissue damage characterized by oedema, vacuolization and detachment of the keratinocytes in all cell layers. Invasion of deeper parts of the epithelium by *C. albicans* was observed at 24 h (Fig. 2a). To investigate whether PMNs (neutrophils, eosinophils or basophils) could reduce or modulate *C. albicans*-induced tissue damage, we added purified PMNs to the model. When PMNs were added to the apical layers of the epithelium 6 and 12 h after infection, *C. albicans* penetration and epithelial damage were significantly reduced compared with the control (PMNs not added) (Fig. 2b). A similar protective effect was noticed when PMNs were added to the basal side of the intact polycarbonate layer of the inverted samples, which prevented direct contact between *C. albicans* and the PMNs. In these experiments, we observed that PMNs were firmly attached to the basal side of the polycarbonate filter (Fig. 2c). Perforation of the filter allowed transepithelial migration of PMNs into the RHE, which also coincided with protection (Fig. 2d, e). In contrast to RHE infected with *C. albicans*, non-infected RHE did not stimulate basal attachment of PMNs to the polycarbonate layer or transepithelial migration of PMNs (not shown). Similar effects were seen in our model when non-opsonized PMNs were used for the experiments.

These marked histological differences in tissue damage corresponded with significant differences of LDH release, which was used as a marker of epithelial cell injury. The release of LDH (U l⁻¹, mean ± SD) of all experiments was separated into classes by the LSD-test (*P*<0.05). LDH values (164.6 ± 18) for SC5314 infection of RHE in the absence of PMNs (class D) were significantly higher than those of all other samples. In the presence of PMNs, LDH values of RHE samples with perforated (58.2 ± 4.2) or with intact filters (49.6 ± 2.7) could not be separated from one another (class C), but were significantly lower than LDH values caused by SC5314 alone. LDH levels of the infected samples after apical addition of PMNs (27.4 ± 4.2) were further reduced (class B), and the lowest LDH release (9.8 ± 2.8) was observed in the uninfected PBS-treated RHE (class A).

Growth inhibition of *Candida* cells by PMNs was analysed by a quantitative plate-count method. Similar percentage inhibition values (12/24 h) were seen when PMNs were added to the apical layers (34 ± 8/44 ± 12%), and to the
Cytokine mRNA expression in response to mucosal *C. albicans* infection in the presence and absence of PMNs (QRT-PCR)

QRT-PCR of uninfected RHE 12 and 24 h after incubation with PBS demonstrated constant basal levels of mRNA expression for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-1α, IL-1β, IL-8, TNF-α and GM-CSF (Table 1). In contrast, *C. albicans*-infected RHE showed a strong increase of IL-8 and GM-CSF expression, and a moderate increase of gene expression for IL-1α, IL-1β and TNF-α after 12 and 24 h. (shown for 24 h in Table 1).

Addition of PMNs to the RHE model of oral candidosis clearly modulated expression of all the investigated cytokines. Interestingly, a further increase of IL-1α, IL-8 and GM-CSF expression was only seen in the experiments enabling transepithelial migration of PMN cells through the infected epithelium; (e) chemotaxis and transepithelial migration of PMNs (arrows) through a pore of the polycarbonate layer of the RHE into epithelial layers and to the surface of the mucosa. Bar (a–d) ~ 65 μm; (e) ~ 50 μm.

**Table 1.** Quantitative analysis of mRNA levels of selected cytokine-encoding genes in non-infected and *C. albicans*-infected epithelial samples in the presence and absence of PMNs

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>mRNA level in the presence of:</th>
<th>PBS</th>
<th>wt</th>
<th>wt, PMN apical</th>
<th>wt, PMN basal</th>
<th>wt, PMN basal, perforated filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td></td>
<td>1·0</td>
<td>230·1±24·3</td>
<td>55·6±7·58</td>
<td>178·86±21·7</td>
<td>838·4±43·8</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td>1·0</td>
<td>29·3±3·5</td>
<td>36·3±7·33</td>
<td>36·7±3·4</td>
<td>48·6±6·2</td>
</tr>
<tr>
<td>IL-1α</td>
<td></td>
<td>1·0</td>
<td>13·1±2·6</td>
<td>8·7±2·4</td>
<td>7·3±2·5</td>
<td>25·2±4·1</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>1·0</td>
<td>21·9±3·7</td>
<td>11·8±1·7</td>
<td>11·2±3·8</td>
<td>12·4±3·3</td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td>1·0</td>
<td>257·7±34·9</td>
<td>145·4±17·5</td>
<td>105·9±6·7</td>
<td>727·1±9·3</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>1·0</td>
<td>0·85±0·2</td>
<td>0·97±0·32</td>
<td>0·89±0·3</td>
<td>0·90±0·1</td>
</tr>
</tbody>
</table>

PBS, non-infected epithelial sample; wt, infection with wild-type *C. albicans*. PMNs were added to the model 12 h after infection. The expression levels of cytokine genes stimulated by *C. albicans* SC5314 after 24 h were related to the expression level of non-infected epithelia 24 h after incubation with PBS (1·0). Five independent experiments were analysed. Values show mean ± SD.
Table 2. FACS analysis of cytokine release 24 h after infection of epithelial cells with C. albicans in the presence and absence of PMNs

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>PBS</th>
<th>WT</th>
<th>WT, PMN apical</th>
<th>WT, PMN basal</th>
<th>WT, PMN basal, perforated filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>1.9±0.8</td>
<td>10.3±2.3</td>
<td>12.3±5.1</td>
<td>13.5±8.5</td>
<td>18.3±5.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.8±0.2</td>
<td>2.9±0.5</td>
<td>2.8±0.9</td>
<td>3.5±1.5</td>
<td>4.9±1.7</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.5±1.3</td>
<td>7.2±1.4</td>
<td>2.5±0.6</td>
<td>2.8±0.9</td>
<td>2.3±1.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>20.4±2.5</td>
<td>32.5±4.3</td>
<td>30.7±9.0</td>
<td>42.4±9.3</td>
<td>66.2±9.3</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.3±2.1</td>
<td>2.5±1.9</td>
<td>2.4±0.5</td>
<td>1.4±0.4</td>
<td>4.8±5.4</td>
</tr>
<tr>
<td>IL-8</td>
<td>260.5±23.1</td>
<td>501.5±20.8</td>
<td>487.5±35.2</td>
<td>512.3±42.3</td>
<td>4243.7±87.3</td>
</tr>
</tbody>
</table>

Cytokine secretion analysis in response to mucosal C. albicans infection in the absence and presence of PMNs (FACS analysis)

The modulation of the immune response was further characterized at the protein level by FACS analysis of the maintenance medium (Table 2). Non-infected mucosa produced only low levels of IFN-γ, TNF-α and IL-1β, moderate levels of IL-6 and IL-10, but high IL-8 levels. In response to infection with C. albicans in the absence of PMNs, epithelial cells produced increasing concentrations of IFN-γ, TNF-α, IL-1β, IL-6 and IL-8, whereas IL-10 secretion was not affected by epithelial infection. Addition of PMNs stimulated IFN-γ and TNF-α production and reduced IL-10 secretion in all samples, while further increases in IL-1β, IL-6 and IL-8 production were only observed during transepithelial migration of the PMN cells.

DISCUSSION

Mucosal epithelial cells provide an effective innate host defence mechanism against C. albicans (Steele et al., 2000). Furthermore, PMNs are frequently found at sites of Candida infection, and this is thought to be a hallmark of oral candidosis in humans (Challacombe, 1994; Eversole et al., 1997). Anticandidal activity and cell-mediated immunity mediated by epithelial cells and PMNs might represent the most important line of host defence to prevent superficial Candida infections (Farah et al., 2002; Fidel, 2002a; Kullberg et al., 1999). Several studies have analysed the role of PMNs in local defence by monoclonal depletion in murine models of vaginal candidosis (Black et al., 1998; Fidel et al., 1995, 1999; Fulurija et al., 1996). However, most data from these studies demonstrated no significant role for PMNs in resolving C. albicans vaginal infections. The role of PMNs in protection against oral candidosis has not been sufficiently investigated. A recent study using two mouse models showed a significant contribution of PMNs in resolving an oral infection in only one of the two models (Farah et al., 2001). The bioassay system presented in our study demonstrates infection-induced chemoattraction of PMNs, which are able to migrate transepithelially to the site of C. albicans infection. Moreover, the in vitro model enables the direct and indirect effects of the PMNs in protection against oral Candida infections to be studied.

Recently published studies have shown induction of IL-8 synthesis in keratinocytes after stimulation with C. albicans (Dongari-Bagtzoglou & Kashleva, 2003; Schaller et al., 2002). Since attachment and/or transepithelial migration of PMNs in this study was only seen after epithelial infection and concomitant strong expression of the chemotactic cytokines GM-CSF, IL-6 and IL-8, we concluded that these cytokines contributed to the transepithelial migration of PMNs in our model. In vivo, PMNs accumulate rapidly at the site of infection in the oral cavity as a result of host signals (Borish et al., 1989; Djeu et al., 1990). IL-6 and IL-8 act as potent chemoattractants and promote degranulation and other antifungal activities of PMNs (Djeu et al., 1990; Kullberg et al., 1999). GM-CSF is known as a potent neutrophil growth factor, stimulating cytokine production and fungicidal activity of leukocytes (Tansho et al., 1994). Therefore, our RHE bioassay system seems to accurately imitate oral candidosis in vivo.

Infection of RHE with C. albicans also induced expression of IFN-γ and TNF-α, which are linked with a protective Th1 response during systemic infection (Romani, 1999). In contrast, IL-10, which is associated with a Th2-mediated susceptibility to candidosis (Romani, 1999), was down-regulated in our bioassay system. PMNs, especially those migrating into epithelial tissue, enhanced this Th1 up- and Th2 down-regulation pattern and protected the mucosa against C. albicans infection by inhibiting fungal growth.

These data implicate a protective role for Th1-type cytokine release from epithelial cells during oral candidosis. Protection against tissue damage was observed by direct interaction of the PMN cells with C. albicans, either by apical application or by transepithelial migration of PMNs from the basal side. Interestingly, the protective effect was also seen in a modified experimental assay in which direct physiological contact between PMNs and C. albicans was prevented and only indirect interaction between PMNs,
keratinocytes and C. albicans was possible. These results suggest that immunological cross-talk between epithelial cells and PMNs via cytokines, rather than direct phagocytosis of C. albicans cells by PMNs, may be of prime importance in initiating an epithelial-mediated protective anticandidal immune response in the oral cavity.

In summary, our model of oral candidosis supplemented by PMNs provides an attractive tool for studying the immunological cross-talk between keratinocytes and immune cells, and the chemoattraction of PMNs to the site of infection. Our results suggest an important role for PMNs in clearance of experimental oral candidosis. The protective effect was associated with a Th1-linked immune response, but was not necessarily connected to the direct interaction of PMNs with the pathogen.

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