Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms

Constantin F. Urban,1* Ulrike Reichard,1,2 Volker Brinkmann2 and Arturo Zychlinsky1*
1Department of Cellular Microbiology and 2Microscopy Core Facility, Max Planck Institute for Infection Biology, Schumannstrasse 21/22, 10117 Berlin, Germany.

Summary
Neutrophils phagocytose and kill microbes upon phagolysosomal fusion. Recently we found that activated neutrophils form extracellular fibres that consist of granule proteins and chromatin. These neutrophil extracellular traps (NETs) degrade virulence factors and kill Gram positive and negative bacteria. Here we show for the first time that *Candida albicans*, a eukaryotic pathogen, induces NET-formation and is susceptible to NET-mediated killing. *C. albicans* is the predominant aetiologic agent of fungal infections in humans, particularly in immunocompromised hosts. One major virulence trait of *C. albicans* is its ability to reversibly switch from singular budding cells to filamentous hyphae. We demonstrate that NETs kill both yeast-form and hyphal cells, and that granule components mediate fungal killing. Taken together our data indicate that neutrophils trap and kill ascomycetous yeasts by forming NETs.

Introduction
Human polymorphonuclear neutrophils are one of the first immune cells recruited to an infection site. These cells efficiently engulf and kill microbes (Borregaard and Cowland, 1997; Hampton *et al.*, 1998; Segal, 2005). Upon phagocytosis, neutrophil granules fuse with the phagosome. Engulfed microbes are killed by the ensuing oxidative burst and by the discharge of antimicrobial granule contents into the phagocytic vacuole. Recently we showed that upon activation with phorbol myristate acetate (PMA) or interleukin-8 neutrophils form extracellular fibres that contain chromatin and granular proteins (Brinkmann *et al.*, 2004). The structure of the neutrophil extracellular traps (NETs) is maintained by the DNA because NETs are degraded by DNase but not by proteases (Brinkmann *et al.*, 2004). The NETs contain histones H1, H2A, H2B, H3 and H4 as well as several granule proteins, such as bactericidal permeability increasing protein (BPI), neutrophil elastase and myeloperoxidase. Together NET-associated antibacterial granule proteins and the histones degrade virulence factors and kill bacteria. Indeed, the bactericidal properties of histones have been recognized for decades (Hirsch, 1958; Hiemstra *et al.*, 1993; Kim *et al.*, 2002).

Neutrophils play a crucial role in controlling and clearing fungal infections (Lehrer and Cline, 1969a; Cockayne and Odds, 1984; Mansour and Levitz, 2002), but the role of NETs in controlling eukaryotic pathogens is not known. *C. albicans* is the most common causative agent of mycoses in humans. Candidiasis can range from a mild superficial infection of the skin and mucosa to severe disseminated systemic disease (Naglik *et al.*, 2004; Romani, 2004). Severe candidiasis occurs particularly in immunocompromised individuals. To disseminate in the host *C. albicans* has evolved many different virulence traits. One of these traits is the ability to reversibly switch from singular budding cells (yeast-form cells) to a filamentous form (hyphae). The yeast form is highly proliferative while hyphae are important for invasion and destruction of tissues (Gow *et al.*, 2002; Whiteway and Oberholzer, 2004).

Neutrophils are able to kill *C. albicans* hyphal and yeast-form cells. However, it is poorly understood how killing of filaments is accomplished, because they are too large to be engulfed by a single neutrophil. Here we report that *C. albicans* induces neutrophils to form extracellular traps that capture and kill *C. albicans* hyphal as well as yeast-form cells. We propose that the NETs kill microbes which neutrophils can not phagocytose due to the size of the microbe.

Results
*Candida albicans* induces NETs
We tested whether a eukaryotic pathogen can induce human neutrophils to release NETs. We infected purified naïve neutrophils with opsonized *C. albicans* hyphae
at a multiplicity of infection (moi) of 0.2 (C. albicans : neutrophils) (Fig. 1). After the indicated time points we fixed and stained the samples for DNA, neutrophil elastase (a granular component of the NETs), and C. albicans cell walls. Uninfected neutrophils rarely made NETs (Fig. 1A) even when incubated with serum. Neutrophils fixed immediately upon addition of C. albicans also did not form NETs (Fig. 1B). Ninety minutes after infection, however, neutrophils started to release NETs (Fig. 1C). Longer incubation with opsonized hyphae (3 h) resulted in more NET-formation (Fig. 1D). Furthermore, the number of NETs correlated with the amount of C. albicans added to the neutrophils. Decreasing NET-formation was observed with lower moi (data not shown). Opsonized yeast-form cells induced NET-formation in a similar manner to hyphae (data not shown). These data indicate that opsonized C. albicans is sufficient for induction of NETs.

To investigate whether opsonization of C. albicans is a prerequisite for NET-induction we compared opsonized (Fig. 1A–D) with unopsonized (Fig. 1E–H) hyphae. C. albicans hyphae or yeast (data not shown) alone induced NET-formation, although opsonization seemed to increase the number of NETs. Taken together, these data show that both hyphal and yeast-form cells of C. albicans induce neutrophils to form NETs even without opsonization. This is the first report that a eukaryotic pathogen alone is able to induce NET-formation.

NETs trap C. albicans hyphae and yeast-form cells

As C. albicans induced the release of NETs, we analysed whether NETs and C. albicans interact. We isolated and activated human neutrophils to synchronize the induction of NETs. We infected the neutrophils with both growth forms of C. albicans for 1 h and processed them for scanning electron microscopy (SEM, Figs 2A–D and 3A–C) and immunofluorescence (Fig. 2E). The NETs are fibres with smooth stretches of around 16 nm in diameter and globular domains of around 25 nm that also aggregate into larger threads (Figs 2B, D and 3C). We visualized neutrophils (Fig. 2A, arrow) and C. albicans hyphae (Fig. 2A) and yeast-form (Fig. 3A) in close proximity to NETs (arrow heads).

Neutrophils can attach and spread along hyphae, even though these phagocytes are significantly smaller than a hypha (Diamond et al., 1978). We confirmed these observations and showed that neutrophils engulfed hyphae by entwining around the hyphal filaments (Fig. 2A, arrow). Neutrophils were capable of changing their shape (Fig. 2C). To enclose a hypha completely often several neutrophils lined up next to each other (Fig. 1B, arrows).

Interestingly, we observed that both hyphae (Fig. 2A, B and D, arrow heads) and yeast (Fig. 3A and B, arrow heads) are trapped in NETs. Frequently we found clusters of C. albicans cells associated to the extracellular fibres.
(Figs. 2A and 3A). In high-resolution images we saw that NETs cover the surface of hyphal (Fig. 2B–D) and yeast-form cells (Fig. 3B) as well as that of neutrophils (Fig. 2C). This indicates that NETs bind the eukaryotic microbe *C. albicans*.

After fixation, we immunostained specimens using antibodies against neutrophil elastase (green) and against *C. albicans* antigens (red). DNA was detected by an intercalating dye (blue). A three-dimensional reconstruction of confocal z-stacks shows how a hypha is caught in a NET (Fig. 2E). Moreover, the filament is partially enclosed by a neutrophil nearby the hypha's mother cell. The colocalization of NETs, neutrophils and hyphae is indicated by the yellow colour. This suggests that NET-trapping and engulfment of hyphae act together to control the microbe. The immunostainings for NET-associated proteins, as neutrophil elastase, and the structural characteristics observed in SEM micrographs confirm the identity of the NETs.

Fig. 2. Scanning electron microscopy (SEM) micrographs and 3D-reconstruction of immunofluorescence of hyphae trapped in NETs. Neutrophils were infected with *C. albicans* hyphae. A. NETs cover hyphae (arrow heads) and a neutrophil encloses a hypha (arrow). Engulfment and NET-trapping of hyphae were seen in the same SEM specimen. B–D. High resolution images of (A). (B) NETs containing smooth stretches and globular domains. The fibres attach to the surface of a hypha. Several fibres can aggregate into larger threads. (C) Interface of the neutrophil on the right and the hypha on the left (arrow). The neutrophil largely adjusts to the shape of the hypha. NETs attach to both the hypha and the neutrophil. (D) Hyphae covered by NETs. Bar in (A) 10 µm, bar in (B–D) 1 µm. E. 3D-reconstruction of an immunostaining based on confocal z-stacks. As described in Fig. 1, NETs contain neutrophil elastase (green) and DNA (blue). *C. albicans* is stained in red. A hypha is partially engulfed by a neutrophil and additionally trapped in a NET demonstrating that both, engulfment and NET-trapping can happen at one site and similar time points. Bar in (E) 1 µm.

Fig. 3. SEM micrographs of yeast-form cells trapped in NETs. Neutrophils were infected with *C. albicans* yeast-form cells. A–B. A cluster of yeast-form cells is covered by NETs and NETs attached to yeast-form cells (arrow heads). C. A high resolution image of a section of a NET showing fibres with smooth stretches and globular domains.
Taken together, the microscopical observations show that NETs cover and trap both growth forms of C. albicans, hyphae and yeast-form cells.

**NETs kill C. albicans hyphae and yeast-form cells**

As NET-fibres trap C. albicans hyphae and yeast-form cells we tested whether NETs can kill both forms. Neutrophils were first activated with PMA and then treated with the actin inhibitor cytochalasin D (cyt D) to block phagocytosis and hence measure extracellular killing exclusively. PMA-activation was necessary to measure NET-mediated killing immediately upon addition of C. albicans. Fungal survival was monitored by plating after the indicated times.

Figure 4A and B show the killing of hyphae and yeast-form respectively. Killing was calculated as percentages of control values (Candida colonies after incubation with neutrophils compared with cultures that were incubated in medium alone). Total killing refers to incubation of C. albicans and neutrophils without cyt D and reflects microbial killing in the phagosome as well as extracellularly. External killing is the microbicidal activity in cultures in the presence of cyt D, where phagocytosis was blocked. After a 60 min incubation, 5–10% of yeast-form cells as well as hyphae were killed in the presence of cyt D, and after 2 h, these values increased to 20–30%. In the absence of cyt D, the killing ranged from 60 to 80% for both C. albicans growth forms. This indicates that, under the conditions tested, 20–30% of microbial killing is extracellular.

We demonstrated that the extracellular killing was NET-mediated with three different approaches. First, as DNA is the scaffold of the NETs (Brinkmann et al., 2004), we degraded them using protease-free DNase-1. The enzyme was added at the time of infection and the cultures were not washed after treatment with DNase-1, leaving the overall composition of the sample unaltered. Addition of DNase-1 reduced killing of C. albicans from 85% to 49% (Fig. 4C). This difference (36%) is in the same range as the extracellular killing (24%), suggesting, again, that extracellular killing was mediated by the NETs only. Thus, the fibrous structure of the NETs is most likely necessary for the killing of the yeast.

Second, we showed that DNase-1 does not affect intracellular killing (Fig. 4D). We compared killing rates in the presence and absence of DNase-1. We used naïve neutrophils without PMA stimulation and infected them with C. albicans. Within 1 h of infection these neutrophils do not form NETs. Killing of C. albicans is then exclusively phagolysosomal. In this experiment, addition of DNase-1 did not affect killing rates of C. albicans (Fig. 4D).

Lastly, when phagocytosis was blocked using cyt D and simultaneously, the NETs were degraded by DNase-1, fungal killing decreased to 0% (Fig. 4E). Instead, we noticed a twofold increase in C. albicans colony-forming units (cfu) in the presence of neutrophils that were blocked by cyt D and DNase-1 as compared with the respective samples without neutrophils, even though neither cyt D nor DNase-1 alone affected the number of cfu of C. albicans (data not shown). To investigate whether the increase in cfu was due to disarmed neutrophils we added the supernatant of the same number of lysed neutrophils to C. albicans. Indeed, the cfu also increased almost two-fold (Fig. 4E).

Taken together, these data strongly suggest that extracellular killing of C. albicans hypha and yeast-form cells is dependent on NETs.

**Granular extracts but not histones kill C. albicans**

Neutrophil extracellular traps contain both granular proteins and histones. Mammalian histones can kill bacteria (Hirsch, 1958; Hiemstra et al., 1993; Kim et al., 2002). Histone H2A is able to kill Gram negative bacteria at concentrations as low as 2 µg ml\(^{-1}\) (Brinkmann et al., 2004). Granules contain many antimicrobials, including BPI, lactoferrin and defensins. We tested which NET components were responsible for C. albicans killing. Both hyphal and yeast-form cells were incubated with 0, 20 and 200 µg ml\(^{-1}\) H2A or with a mixture of histones (H1, H2A, H2B, H3 and H4) for 30 min at 37°C. Subsequently serial dilutions were plated and growth was monitored 24 h later. We found that at a concentration of 200 µg ml\(^{-1}\) neither C. albicans hyphae (Fig. 5A, lanes 1–3) nor yeast-form cells (Fig. 5B, lanes 1–3) were susceptible to H2A (Fig. 5A and B, lanes 1–3) as well as to the histone mixture (data not shown). As a control, we showed that more than 80% of a culture of the Gram negative Shigella flexneri and more than 60% of a culture of the Gram positive Staphylococcus aureus were killed at concentrations as low as 5 µg ml\(^{-1}\) H2A in the same assay. Twenty µg ml\(^{-1}\) histone mixture killed 100% of a S. flexneri culture (data not shown). We concluded that histones do not kill C. albicans.

We then tested whether granule proteins had candidacidal activity. Neutrophils were isolated from buffy coats, lysed and sonicated to release the granules. We obtained a human Neutrophil Granular Extract (hNGE) by acid extraction (as described in Experimental procedures). We incubated hyphal and yeast-form cells with hNGE in a similar assay as the one described above and monitored hNGE-mediated fungal killing within 30 min hNGE killed both C. albicans growth forms in a dose-dependent manner (Fig. 5A and B, lanes 4–7). At a concentration of 1 mg ml\(^{-1}\) hNGE (lane 5) fungal growth was already lower than in control cultures. At 6 mg ml\(^{-1}\) C. albicans yeast-form cells and hyphae were killed com-
Fig. 4. Neutrophil extracellular traps kill *C. albicans* hyphae and yeast-form cells. Neutrophils were infected with either hyphae (A) or yeast-form cells (B) and incubated for the indicated time points. Colony-forming units of *C. albicans* yeast-form cells were determined as described in Experimental procedures. External killing was measured in the presence of the actin inhibitor cyt D and total killing including intra- and extracellular killing without cyt D. External killing for both growth forms of *C. albicans* increased from 5–10% after 1 h to 20–30% after 2 h. C. NETs were digested with 100 U ml⁻¹ DNase-1 and neutrophils were infected with *C. albicans*. Addition of DNase-1 reduced fungal killing from 84% to 48%, the difference (36%) is similar to the extracellular killing (24%). D. Using naïve neutrophils that did not form NETs and hence, exclusively kill intracellularly, measured fungal killing was not affected by DNase-1. E. Isolated neutrophils (2 \times 10^6 ml⁻¹), activated with PMA for 30 min (bars 1–4) or lysed (bar 5), were infected with *C. albicans* and the indicated substances were added for 2 h at 37°C. DNase-1 was adjusted to a final concentration of 100 U ml⁻¹ (bar 2), cyt D to 10 \mu g ml⁻¹ (bar 3). Survival is reported as the percentage of Candida colonies after incubation with neutrophils compared with microbial cultures that were incubated in the same medium alone. *C. albicans* formed twice as many cfu (bar 4, over 250%) in the presence of disarmed neutrophils [blocked phagocytosis (cyt D) and NET-mediated killing (DNase-1)] compared with the respective controls without neutrophils. Thus, simultaneous addition of DNase-1 and cyt D to neutrophils completely blocked killing of *C. albicans*. To mimic the presence of disarmed neutrophils lysed neutrophils were added to *C. albicans*. This also increased fungal cfu (bar 5, almost 200%) indicating that *C. albicans* can grow faster in the presence than in the absence of disarmed neutrophils. An moi of 0.01 (*C. albicans*: neutrophils) was used in all experiments.

pletely (lane 7). As a control, 1 mg ml⁻¹ hNGE sterilized a *S. flexneri* culture (data not shown).

These data show that the granular proteins are effective fungicides and are likely to mediate NET-killing of the trapped yeast.

Discussion

Neutrophils are one of the major phagocytes and are a crucial part of innate immunity. These phagocytes are able to form NETs that degrade virulence factors and kill bacteria. Here we showed that the NETs entrap and kill a eukaryotic microbe. Both, yeast-form and hyphal cells of *C. albicans*, are trapped and killed by NETs.

The presence of microbes activates neutrophils. The phagocytes become chemotactic, migrate towards the site of infection and produce an oxidative burst (Borregaard and Cowland, 1997). We demonstrated that opsonized as
well as unopsonized Candida hyphae and yeast-form cells are able to activate neutrophils and induce NET-formation (Fig. 1). We propose that parallel signalling pathways might exist for NET-formation or two different stimuli could converge on one pathway. For opsonized C. albicans Fcy-receptors (Pricop and Salmon, 2002), for unopsonized microbes pattern recognition receptors, such as Toll-like receptors (Netea et al., 2002) or dectin (Brown and Gordon, 2001) may be involved. The elucidation of signalling mechanisms of NET-induction will help us to understand the process of NET-formation which is still unknown.

Released NETs seem to attach to the microbial cell wall. Although the composition of bacterial and fungal cell walls is different, we found that C. albicans as well as Gram positive and negative bacteria are caught by NETs. It is possible that the NETs have specific recognition sites for different microbes. However, the microbial attachment to the NETs is more likely mediated by ionic forces. Thus, we speculate that potentially any negatively charged surface can be bound by NETs. The mannan side-chains of yeast cell wall proteins contain numerous phosphodiester bridges (Klis et al., 2001), so the cell walls can act as an ion exchanger that efficiently binds positively charged ions and proteins. Many granule proteins and all histones are highly cationic (positively charged), suggesting that the NETs actually attach ionically to the surface of C. albicans, as well as many different ascomycetous yeasts, because their cell wall architecture is very similar (De Groot et al., 2005). However, we do not know the nature of the forces which mediate microbial attachment to the NETs.

Previous reports showed that human neutrophils kill C. albicans hyphae as well as yeast-form cells (Cockayne and Odds, 1984; Edwards et al., 1987; Christin et al., 1997; Du et al., 2005). Here we show that in vitro, this killing is mediated by both phagocytosis and by the NETs. Between 20% and 30% of the candidacidal activity of neutrophils is extracellular and the rest is phagolysosomal.
Median killing (4–6 mg ml\(^{-1}\)) is very similar, reflecting their similar susceptibility to NETs. The NETs were gel overlay and radial diffusion assays. Whether histones have candidacidal activity under different conditions remains to be determined. Nevertheless, our data indicate that histones are unlikely to play a major role in killing C. albicans, but their cationic properties might be important in promoting binding of the fungal pathogen to the NETs.

Granular extract, however, killed C. albicans hyphae and yeast-form efficiently (Fig. 5A and B). The susceptibility of hyphal and yeast-form cells towards hNGE was very similar, reflecting their similar susceptibility to NET-mediated killing (4–6 mg ml\(^{-1}\)). The antifungals present in the granule extract are likely to include lactoferrin (Lupetti \textit{et al.}, 2000) and myeloperoxidase (Lehrer and Cline, 1969b; Wright and Nelson, 1985). In contrast, a \textit{S. flexneri} culture was sterilized by 1 mg ml\(^{-1}\) hNGE. This might simply be due to the fact that the susceptibility of microbes to hNGE differs largely. Furthermore, both oxygen-dependent (Wysong \textit{et al.}, 1989; Thompson and Wilton, 1992) and -independent mechanisms (Reeves \textit{et al.}, 2002; Newman \textit{et al.}, 2005) or the collaborative action of both (Hiit \textit{et al.}, 1999) have been reported to be important for candidacidal activity of neutrophils underscoring the importance of oxidative killing mechanisms. However, in this study we focused on NET-mediated killing of C. albicans. The close association of the microbe and the NETs could provide the local concentration of granular proteins required to kill C. albicans.

Neutrophils are continuously recruited to the site of infection and are activated by different cytokines and also by C. albicans (Fig. 1). We propose that NETs formed by recruited neutrophils prevent the spread of and kill the pathogens. \textit{In vitro} we showed that neutrophils capture yeasts in two ways: By phagocytosis and by NET-trapping. The NETs could particularly support killing of microbes that are too large to be phagocytosed efficiently. A neutrophil can circumvent this problem by enclosing microbes together with other neutrophils (Fig. 1B and Diamond \textit{et al.}, 1978). However, the NET-killing could represent one mechanism how large pathogens are killed by neutrophils.

Neutrophils are an essential part of the innate immune response that clear fungal infections (Mansour and Levitz, 2002). As C. albicans induces NET-release and NETs trap as well as kill C. albicans, we propose that NETs are relevant \textit{in vivo}. Additionally, NETs have been visualized \textit{in vivo} previously, for example in human spontaneous appendicitis (Brinkmann \textit{et al.}, 2004). However, \textit{in vivo} models with impaired NET-formation do not exist to test this hypothesis. One problem is that knocked-down granule components would hamper not only NET-mediated but also phagolysosomal killing. Thus, a major challenge for future work will be to elucidate the \textit{in vivo} relevance of NETs by understanding the mechanism of NET-formation.

**Experimental procedures**

**Strains and media**

\textit{Candida albicans} clinical isolate SC 5314 was cultured overnight in YPD (1% yeast extract, 2% bacto peptone, 2% glucose) at 30°C. For all experiments C. albicans was subcultured at an \textit{OD}\(_{600}\) of 0.1 either in RPMI containing 2% glucose at 37°C to induce hyphae or in YPD at 30°C to induce yeast-form for 4 h. S. \textit{flexneri} M90T was used as a control for the antimicrobial properties of histones and granular extracts. S. \textit{flexneri} was grown to the exponential phase at 37°C with aeration in tryptic soy broth.

**Isolation of neutrophils**

Human neutrophils were isolated from peripheral blood of healthy donors using dextran-Ficoll (Weiss \textit{et al.}, 1985).

**Scanning electron and confocal immunofluorescence microscopy**

In microscopy assays 4 × 10\(^5\) neutrophils per sample were allowed to settle on glass coverslips treated with 0.001% polylysine.
For immunostaining we used RPMI medium containing 2% inactivated human serum for samples with opsonized and medium containing 2% human serum albumin for samples with unopsonized C. albicans. After addition of C. albicans (moi 0.2), sample plates were centrifuged for 3 min at 700 g and incubated at 37°C. Incubation time points were 5, 90 and 180 min. The uninfected controls were incubated for 180 min in the absence of C. albicans. After incubation samples were fixed with 4% PFA, blocked overnight (10% normal goat serum, 5% cold water fish gelatin, 1% bovine serum albumin, 0.05% Tween-20 in PBS) and incubated with primary antibodies. These antibodies were directed against human neutrophil elastase (monoclonal, produced in house) and various C. albicans antigens (polyclonal, rabbit, Acris BP1006). Primary antibodies were detected using secondary antibodies coupled to Cy2 and Cy3 respectively. For DNA detection, DRAQ5 was used. Specimens were analysed with a Leica TCS-SP confocal microscope (Bensheim, Germany).

For SEM, neutrophils were activated for 30 min using PMA to synchronize the infection. The medium was replaced with RPMI containing 2% heat inactivated human serum and C. albicans was added at the indicated moi. Samples were fixed with 2.5% glutaraldehyde, post-fixed using repeated incubations with 1% osmium tetroxide/1% tannic acid, dehydrated with a graded ethanol series, critical-point dried and coated with 5 nm platinum. Due to the fragility of the NET-structures, disturbances of the media in each step were kept to a minimum to preserve the structures. After dehydration and critical-point drying, the specimens were coated with 5 nm carbon and analysed in a Leo 1550 scanning electron microscope (Oberkochen, Germany).

In vitro killing assay

Assays were performed as described previously (Brinkmann et al., 2004). Briefly, human neutrophils were resuspended at 2 × 10⁶ ml⁻¹, and allowed to adhere to plastic plates in RPMI medium with or without addition of 25 nM PMA (Sigma). After 30 min incubation at 37°C, the medium was carefully replaced with serum-free RPMI, containing 2% heat inactivated pooled human serum with or without cyt D (10 µg ml⁻¹) and incubated further for 20 min before infection with C. albicans. Cyt D (Sigma) treatment did not affect NETs and this concentration was effective in blocking fungal phagocytosis. Prior to infection of neutrophils C. albicans was subcultured for 4 h either in RPMI at 37°C to induce hyphae or in YPD at 30°C to induce yeast-form cells. To show that NETs were important in extracellular killing, samples were pre-incubated with 100 U ml⁻¹ of RNase and protease-free DNase-1 (Worthington) to degrade NETs before the addition of C. albicans (2 × 10⁴ ml⁻¹). Samples were centrifuged at 700 g for 10 min and incubated at 37°C and 5% CO₂ for 1 and 2 h. C. albicans killing was measured as percentages of control values (C. albicans incubated without neutrophils). The applied moi was 0.01 (C. albicans: neutrophils). Each value was the mean of a triplicate. Yeast-form cell concentration was determined by optical density measurement at 600 nm wavelength (OD₆₀₀) correlating 3 × 10⁵ cells to 1 OD₆₀₀. Hyphae were counted in a Neubauer chamber. To overcome the problem of hyphae clumping, six values were measured for each condition. To alleviate the distortion caused by extreme values the trimmed mean was calculated omitting the highest and lowest values. Additionally, samples were diluted such that 200–500 cfu resulted per plate.

Purification of neutrophil granular extract

Granular extracts (hNGE) were obtained by the sulphuric acid extraction of whole cells according to Weiss et al. (1978). Briefly, neutrophils from buffy coats were isolated as described above, resuspended in cold distilled water and homogenized vigorously by sonication until the samples were white and milky. Granular proteins were extracted with 0.16 N sulphuric acid at 0°C with periodic shaking. Precipitates were removed by centrifugation for 20 min at 16,000 g (4°C). The supernatants were dialysed in Spectra/Por 3.5Kd membrane against 2 l of 20 mM sodium acetate buffer pH 4 for 2 days at 4°C with two changes. The protein concentration of the hNGE prepared for this study was adjusted to 10 mg ml⁻¹.

Fungicidal activity

Candida albicans (2 × 10⁴) were incubated with purified granule extract (hNGE), purified H2A or purified complete histone (Roche Diagnostics) in a total volume of 50 µl for 30 min at 37°C with shaking in Hanks Balanced Salts Solution (HBSS-) containing 10 mM Hepes pH 7.4 and 0.3% Casamino acids. Five microlitre aliquots of serial dilutions (1:3) were spotted onto YPD agar and incubated for 24 h at 30°C.

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