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Candida albicans Grown in Glucose-free Media Contains Serum-independent Chemotactic Activity

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Infection of skin with *Candida albicans* is usually followed by infiltration of neutrophil granulocytes (PMN). So far, chemotaxins for PMN have been isolated from *C. albicans* cultures grown in the presence of glucose. However, since glucose is not present in skin in vivo, a contribution of such factors to Candida-triggered cutaneous inflammation would appear unlikely. In order to clarify this question, chemotactic activity was measured in extracts from three different strains of *C. albicans* which were grown in five different peptone media free of carbohydrates and serum. In addition, four culture systems were supplemented with lipids normally present in human stratum corneum, including a triglyceride, cholesterol, and sphingomyelin. In all sugar-free grown cultures, serum-independent chemotactic activity was detected by use of the Boyden chamber technique. Since, as shown here in vitro, production of neutrophil chemotaxins by *C. albicans* is independent of glucose-feeding, a possible role of Candida-chemotaxins in the pathophysiology of cutaneous candidosis can no longer be excluded.

Key words: Leukotaxins; Polymorphonuclear leukocytes; Lipids; Candidoses.

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Polymorphonuclear leukocytes (PMN) are important effector cells in the containment of infections caused by *Candida albicans* (1, 2, 3). Therefore factors attracting PMN towards the site of infection are of special interest in limiting the disease. Chemotaxinogenic mannans and serum-independent chemotaxins derived from *C. albicans* are in fact able to initiate a chemotactic response of PMN in vitro (4–7). Concerning superficial cutaneous candidosis however, the clinical relevance of such factors appears doubtful, because the stratum corneum usually contains neither complement which can be activated by chemotaxinogens, nor sufficient amounts of glucose (8) which was found to be an essential nutrient of *C. albicans* to produce chemotaxins (5).

Our study was designed to investigate the role of glucose feeding for the production of chemotaxins by *C. albicans*. For this purpose we cultivated *C. albicans* on sugar-free growth media which, however, contained added lipid materials that are natural constituents of human stratum corneum (9) and can be metabolized by *C. albicans* (10, 11, 12). Extracts of the Candida cells grown under these conditions were then tested for direct neutrophil chemotactic activity in a serum-free assay by use of the Boyden-chamber technique. Our finding that production of chemotaxins by *Candida albicans* is not dependent on glucose draws attention to leukotaxins as possible mediators in the pathogenesis of cutaneous candidosis.

MATERIAL AND METHODS

Cultivation of Candida albicans

Three randomly chosen clinical isolates of *C. albicans* (strains 1, 2, and 3) were identified by their morphology on rice-agar and formation of germ-tubes. This was followed by cultivation on a sugar-free agar consisting of Neopeptone (Difco) 2% in phosphate-buffered saline (PBS). After 7 days, cells from these cultures were suspended in PBS with a density of one million cells per ml and used for inoculation of the following media, called *a*, *b*, *c*, *d*, and *e*: medium *a* consisted of Neopeptone 8 g in 1000 ml PBS, and medium *b* was additionally enriched with 16 ml Tween 80. To medium *b*, 1 g of cholesterol (Sigma no. C-8378) (medium *c*), 1 g of sphingomyelin (Sigma no. S-0756) (medium *d*) or 1 g of tripalmitin (Sigma no. T-8127) (medium *e*) was added. All media were autoclaved, adjusted to pH 7.3 and supplemented with penicillin and chloramphenicol. 30 ml of medium was inoculated with 1 ml of the cell suspension mentioned above. Each strain of *C. albicans* was grown in each medium and all cultures were made in duplicate. Cultures were incubated at 32°C for 7 days, after which the pH of the media had fallen to 6.8–7.1. Contamination was excluded by control cultures on blood-agar for bacteria and on Sabouraud-agar for fungi. The Candida cells were centrifugated, washed three times with 1% Tween 80 in PBS and pure PBS and stored at −20°C until further processing.

Preparation of Candida extracts

Candida cells were ground mechanically with glass beads (diameter 0.75 mm) in a cell disintegrator (Ima GmbH, FRG) at 4000 rpm for 20 min at 4°C. The supernatant of the centrifugated homogenate was filtered through Sep-Pak^R-C-18-cartridges (Waters GmbH, FRG), to which apolar substances are bound. These were then eluted with 4 ml of methanol. 100 µl of the eluate, mixed with 10 µl PBS containing 0.1% (w/v) bovine serum albumin (BSA), was lyophilized and reconstituted with 500 µl of the same buffer for chemotaxis assays. In these experiments, dilutions of 1 : 1, 1 : 2, 1 : 5, 1 : 10, 1 : 50, and 1 : 100 of the reconstituted Candida extracts were used.

Polymorphonuclear granulocytes

Venous blood from healthy human volunteers was centrifugated with Ficoll^R, followed by sedimentation in a gelatin gradient as described earlier (13). In brief, erythrocytes and PMN-rich pellets were mixed with warm gelatin solution 2.5% (w/v) in 0.9% NaCl and erythrocytes were allowed to sediment for 30 min at 37°C. PMN-rich supernatants were collected and contaminating red cells lysed by 0.85% (w/v) NH_4Cl for 10 min at room temperature. PMN were washed twice and finally suspended in PBS containing 0.9 mmol $CaCl_2$, 0.5 mmol $MgCl_2$, and 0.1% (w/v) BSA. Purity of PMN was greater than 97% with more than 92% neutrophils and 5% eosinophils. Viability exceeded 97% by trypan blue dye exclusion and remained so after incubation of PMN with Candida extracts for 1 h at 37°C.

Chemotaxis assays

Chemotactic activity was measured in vitro by use of a modified Boyden-chamber technique (13). The upper compartments were filled with 100 µl suspension of PMN ($2 \cdot 10^6$ cells/ml) and separated by polycarbonate filters (Nuclepore GmbH, FRG, pore diameter 3 µ) from the lower compartments containing the Candida extracts. Chambers were incubated at 37°C for one hour under saturated humidity. Negative controls (buffer solutions containing 0.1% BSA, extracts

Table I. *Chemotactic indices of extracts from Candida albicans strains 1, 2, and 3*

Dilution of C.extr.	Strain no.		
	1	2	3
$\bar{x}$ 1:1	1.43	1.25*	1.37
s	0.16	0.30	0.21
$\bar{x}$ 1:2	1.39	1.55	1.36
s	0.12	0.37	0.19

Values in the table are arithmetic means ($\bar{x}$) of chemotactic indices with their standard deviations (s). Each strain was grown in five different media (*a, b, c, d,*and *e,* see main text), and each experiment was done in triplicate (n=15). Chemotactic indices were significantly greater than 1 ($p \leq 0.001$ for all but one index, marked by *, with $p \leq 0.05$). For extract preparation and chemotaxis assays, see main text.
C.extr. = Candida-extract.

Table II. *Chemotactic indices of extracts from Candida albicans grown in media a, b, c, d,* and *e*

Dilution of C.extr.	Growth medium				
	a	*b*	*c*	*d*	*e*
$\bar{x}$ 1:1	1.21*	1.36*	1.51#	1.40+	1.29*
s	0.19	0.29	0.14	0.26	0.24
$\bar{x}$ 1:2	1.39#	1.39#	1.44+	1.49+	1.47#
s	0.20	0.19	0.37	0.32	0.19

Values in the table are arithmetic means ($\bar{x}$) of chemotactic indices with their standard deviations (s). In each medium (for composition of media, see main text), three different strains (nos. 1, 2, and 3) were grown, and each experiment was done in triplicate (n=9). Indices were significantly greater than 1 with $p \leq 0.05$, ≤ 0.01 or ≤ 0.001 (marked by *, + and # resp.) For extract preparation and chemotaxis assays, see main text.
C.extr. = Candida-extract.

from incubated growth media) and positive controls (using formyl-methionyl-leucyl-phenylalanine, FMLP, Sigma) were measured separately.

Quantification of migrated PMN

PMN which had passed the membrane of the Boyden chamber during the experiments were treated for 10 min with 0.1% Triton X–100 to release β-glucuronidase, as described previously (13). 100 µl of the obtained lysate was incubated with the same amount of 0.01 M *p*-nitrophenyl-β-glucuronide and 0.1 M sodium acetate buffer, pH 4.0 for 18 h, after which time the enzymatic reaction was stopped by adding 100 µl of 0.4 M glycine buffer, pH 10.0. *p*-Nitrophenolate was measured photometrically at 405 nm (photometer SLT 210 Kontron). Lysates of known numbers of PMN ($0.05 - 1.6 \cdot 10^5$ cells) were treated equally for calibration. Chemotactic activity was expressed as chemotactic index (CI), which is defined as the number of migrated PMN in the presence of a stimulus divided by the number of randomly migrated cells (CI = stimulated migration : random migration).

Reproducibility and statistics

All experiments were performed in triplicate, and all measurements were made in duplicate at least. The values presented are arithmetic means with their standard deviations. For statistical analysis, Student's *t*-test was used.

RESULTS

Chemotactic activity was detected in each of the three *Candida albicans* strains grown separately in five different media.

Table I shows chemotactic indices separately listed for extracts of individual strains nos. 1, 2, and 3 in dilutions 1 : 1 and 1 : 2. In this Table the means for each strain grown on all five media (*a, b, c, d,* and *e*) are presented. With each experiment done in triplicate, one mean value stands for 15 different experiments (n=15). All CIs are significantly greater than 1 ($p \leq 0.001$ in all cases but one with $p \leq 0.05$), indicating a positive chemotactic response. Differences between the strains appear to be of only minor importance.

Table II shows the mean values of CIs obtained with extracts from all three *C. albicans* strains diluted 1 : 1 and 1 : 2, separately for the five different media *a, b, c, d,* and *e* in which they were grown. Since all experiments were made in triplicate, each figure is a mean of 9 separate experiments (n=9). Obviously all growth media were sufficient to induce *C. albicans* derived chemotactic activity, as reflected by CIs clearly above 1 (values for p ranging from ≤ 0.05 to ≤ 0.001, see Table II). CIs obtained from yeasts grown in medium *a*, containing neopeptone only but neither glucose nor lipids, appear to be slightly smaller than those from *C. albicans* grown in lipid-

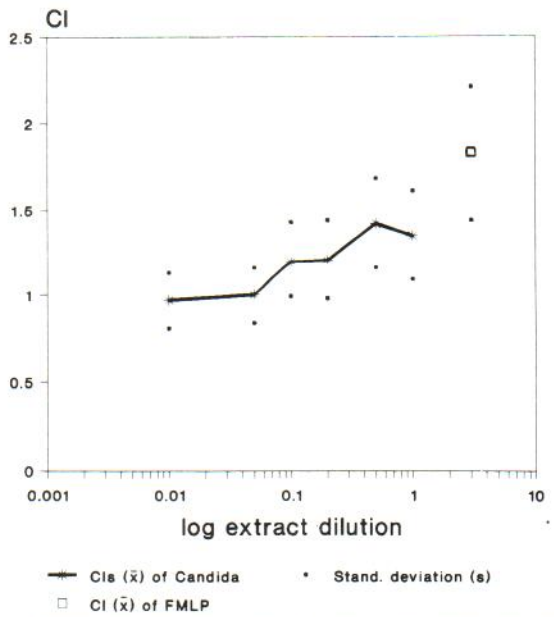

Fig. 1. Chemotactic indices (CIs) of *Candida albicans*; arithmetic means. One asterisk represents results from 3 different strains of Candida albicans (nos. 1, 2, and 3) grown on 5 different media (*a, b, c, d,* and *e,* see main text) each, and each experiment was done in triplicate (n=45). Extracts were diluted 1 : 1, 1 : 2, 1 : 5, 1 : 10, 1 : 50, and 1 : 100 for chemotaxis assays. CIs of extracts diluted 1 : 50 and 1 : 100 are not different from 1, whereas CIs of lower extract dilutions are significantly higher than those ($p \leq 0.001$). CI of FMLP $5 \cdot 10^{-8}$M (positive control) is indicated by open square on the right (arithmetic mean, n=9).

supplemented media *b*, *d*, *e*, and especially in medium *c*, which contained cholesterol. CI of extract from Candida grown on medium *c* and diluted 1 : 1 was significantly higher than the corresponding CI obtained from medium *a* ($p \leq 0.001$).

Chemotactic activity decreased at higher dilutions of the extracts and was undetectable at dilutions of 1 : 50 and 1 : 100 with CIs near 1. No chemotactic activity was detectable in control systems containing pure growth media. This dose-response relationship of chemotactic activity is expressed by Fig. 1, which shows arithmetic means of CIs obtained from all three *C. albicans* strains grown in all of the five media, thus comparing chemotactic activity of the different extract dilutions. Each point in this figure represents 45 different experiments ($n=45$). CIs of extracts diluted 1 : 50 and 1 : 100 did not differ from the negative control, whereas lower dilutions of Candida extracts yielded significantly higher CIs as compared with those ($p \leq 0.001$).

DISCUSSION

Our results show clearly that glucose is not an essential nutrient for the induction of *C. albicans*-derived chemotactic activity. None of our growth media contained carbohydrates, but chemotactic activity was nevertheless detected in extracts of all of our *C. albicans* cultures. Even alimentation with neopeptone alone was sufficient for this. These findings differ from the results of previous studies performed by Cutler (5).

Since human stratum corneum cannot supply glucose, independence from glucose-feeding is an obligatory condition for a possible relevance of these factors in superficial cutaneous candidosis. Under such conditions, these neutrophil chemotactic substances now appear to be interesting as potential mediators for the accumulation of PMN, typical of cutaneous candidosis (14). This view is supported by recent findings showing that attraction of PMN during cutaneous Candida infections can occur without activation of complement (15).

Possibly the addition of lipids may increase the amounts of chemotactic factors in the yeast cells. Since larger amounts of nutrients result in a better growth of the yeast, thus yielding a greater cell mass, this may also be a non-specific effect. No attempt to correlate chemotactic activity with fungal weight was made, as the latter had not been measured exactly.

Since no serum was used in our assays, a chemotactic stimulus indirectly released by activating complement as described for mannans (4, 16) can be excluded. In contrast, our results are consistent with the presence of low molecular weight chemotaxins as previously described by Cutler (5). This author was able to produce chemotaxins from *C. albicans* cultures when the organisms were grown in media containing glucose, which was omitted from the growth media used here. Possible explanations are the short incubation time used by Cutler (5), which in the absence of glucose may prove inadequate for the production of chemotaxins, or differences in the methods for determination of chemotactic activity.

The chemical nature of the chemotaxins remains to be determined. Binding to apolar C-18 Sep-Pak^R-cartridges and solubility in methanol are suggestive of lipids, but an exact analysis is yet to be done. In a previous study (7) we were able to show that the migration of PMN towards *C. albicans* extracts prepared by the same procedure as used here was in fact chemotactic. Furthermore, we could show by cross-desensitization experiments with known chemotaxins, e.g. leukotriene B_4, platelet activating factor and FMLP, that the chemotaxins present in *C. albicans* extracts are biologically different from those mentioned above. In fact there is accumulating evidence for a new class of Candida-derived chemotaxins that may be produced under growth conditions similar to those present in vivo. Because of their possible clinical relevance, purification and isolation of these chemotaxins is desirable and currently in progress in our laboratory.

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