MYCOLOGY

Non-opsonic phagocytosis of *Trichophyton mentagrophytes* arthroconidia by human neutrophils *in vitro*

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Summary. A non-opsonic mechanism of binding and phagocytosis by human neutrophils of *Trichophyton mentagrophytes* arthroconidia is described. This was in direct contrast to the complement dependency of *Candida albicans* phagocytosis. Both serum complement and specific antibody to *T. mentagrophytes* promoted maximal phagocytosis (61 % and 40 % of neutrophils, respectively, contained arthroconidia). Increasing the ratio of arthroconidia to neutrophils did not increase non-opsonic phagocytosis (18–26 %). Phagocytosis of arthroconidia exposed to trypsin in the absence of opsonin was not affected (18 %). However, proteinase and chitinase reduced the level of non-opsonic and opsonic phagocytosis to negligible levels (6·3 % and 4·5 %, respectively). When mannose was added to neutrophils, mannose receptors on the phagocyte membrane were partially blocked when arthroconidia were opsonised, but this did not reduce the level of non-opsonic phagocytosis. The non-opsonic mechanism proposed here may have direct relevance in skin sites poor in opsonins.

Introduction

The mechanisms that defend the host against dermatophyte infections can be divided arbitrarily into local defences, such as the skin, and systemic defences, such as the non-specific and specific immune systems.¹ The skin is a tough, resistant, multi-layered structure whose main function is protection. The epidermis is proliferating constantly and corneocytes are shed from its surface. For dermatophytes to establish an infection, the fungus must adhere and germinate rapidly.^{2,3} The growth of the fungus must keep pace with the rate of epidermopoiesis and exfoliation.

Antibody responses occur both in inflammatory and non-inflammatory types of infection. Antibody appears to have no protective effect in dermatophytosis. Systemic immunological responses induced by dermatophytes are both antibody- and cell-mediated. The non-inflammatory system is the primary defence of the non-immune host because it does not require previous exposure to the invading organism for activation and can respond immediately to protect the host against an invading fungus.

A very early event in the host reaction to invasion by

dermatophytes is the infiltration of neutrophils and epidermal oedema,⁴ and to a lesser extent accumulation of monocytes in the epidermal infiltrate—probably in response to chemotactic components, e.g., C5a, *via* the alternative complement pathway.^{5,6} The accumulation of phagocytes in the epidermal infiltrates is believed to play an important role in the initial host defence to the invasion of dermatophyte germlings. The precise role played by chemotactic epidermal cytokines such as interleukin 8 in the accumulation of neutrophils at the site of dermatophyte infection is not clear.⁷ Dermatophytes are damaged by toxic oxidative products produced by neutrophils and are destroyed by neutrophils extracellularly.^{6,8,9-10} Previous studies have used saprophytic microconidia only.^{9,10} Arthroconidia are the only infective conidia produced under parasitic conditions and are the natural pathogenic elements of dermatophytes since they are the only form of conidia found in tissues.¹¹ Current understanding of the interaction between human effector cells and dermatophytes is very limited.

In the present study the interaction between human neutrophils and arthroconidia of *Trichophyton mentagrophytes* was characterised in a monolayer assay. The non-opsonic mechanism proposed may have direct relevance in skin sites which are poor in opsonins.

Materials and methods

Organisms and stock culture

One strain of *T. mentagrophytes* (strain 126) which had been preserved on silica gel at 4°C was used. Initially, it was recovered from silica gel by growth on glucose peptone agar (GPA) at 28°C. Subcultures were maintained on GPA and incubated at 28°C for 10 days to obtain an optimal yield of fungal growth.

Production and preparation of arthroconidia

Arthroconidia were prepared as pure suspensions of separated cells from 10-day-old cultures grown as surface lawns on glucose 4%, peptone 1% agar in an atmosphere of CO₂ 10%, air 90% at 28°C in an incubation module (Flow Laboratories, Irvine), and flushed through daily with CO₂ 10%, air 90%. Surface growth was harvested from the culture plate with a scalpel blade, suspended in phosphate-buffered saline (PBS), pH 7.2, and agitated for 5 min. The suspension was filtered through column chromatography grade glass wool to remove chains of arthroconidia, washed three times by agitation in PBS followed by centrifugation at 300 *g* for 3 min. The spore suspension was adjusted to a final concentration of 1×10^7 /ml in PBS. This procedure resulted in a pure, uniform arthroconidial suspension of single cells. No obvious damage to arthroconidia occurred. Viability of singlet arthroconidia was determined by incubation in Sabouraud's dextrose broth for 16 h at 37°C. An arthroconidium was considered to have germinated when a visible germ tube had developed. The percentage germination for the strains was 97.00 SD 1.89%.

Preparation of neutrophil monolayers

Monolayers of glass-adherent neutrophils were prepared as described previously.¹² Briefly, drops of blood from finger tip punctures (a single donor was used throughout) were placed on 16-mm diameter coverglasses. The blood drops were incubated for 30 min at 37°C in a humidified chamber. They were then immersed in saline 0.85% w/v at 37°C and agitated to remove adherent red cells. The coverglasses were then washed again in saline, resulting in confluent neutrophil monolayers.

Assessment of phagocytosis

Neutrophil monolayers were placed in the wells of "Repli" (Sterilin) tissue culture plates containing 1 ml of 1×10^5 conidial suspension. Normal human serum (100 µl) was added to each monolayer immediately before incubation at 37°C for 30 min. The monolayers were then washed twice in saline 0.85% and inverted over glass cavity slides. Monolayers were examined within 5 min under high power ($\times 100$ objective) oil immersion microscopy. Ten randomly selected fields of view were examined; the only constraint being that

at least two neutrophils were present in each field. The total number of neutrophils per field was counted and the number of neutrophils ingesting one or more conidia was recorded. From these observations, the percentage phagocytosis (number of neutrophils with one or more ingested conidia/total neutrophil number) was calculated.

Exposure of arthroconidia to trypsin, proteinase and chitinase

To determine the contribution of outer cell wall components to neutrophil binding and phagocytosis, arthroconidia were exposed to various site-specific cell wall solubilising enzymes and the treated spores were added to standard neutrophil monolayer assays. Trypsin (Sigma) was prepared as a 0.25% solution in PBS and used to reconstitute a pellet of arthroconidia (3×10^6 arthroconidia/ml). The spore suspension was incubated for 60 min at 37°C and then washed three times. Proteinase (1 mg; Sigma) was added to the same concentration of arthroconidia and incubated for 18 h at 37°C. Chitinase (1 mg; Sigma) was used in a similar way to that described for proteinase.

Blocking of mannose receptors on neutrophils

D(+) mannose in PBS (25 mg/ml) was added to neutrophil monolayers for 60 min at 37°C. The mannose solution was then removed and arthroconidia were added as for a standard monolayer assay.

Statistical analysis

All data were analysed by Student's *t* test. As replicate experiments were performed on different days and at different times of day, the test results were compared against the control values obtained at the same time. The pooled variance and statistical significance for all control and test values were calculated to overcome the problem of day to day variability.

Results

Phagocytosis of arthroconidia

Non-opsonic ingestion of *T. mentagrophytes* arthroconidia by human neutrophils was clearly evident over a range of arthroconidial concentrations although at a lower level than when serum complement was present in the assay (table I). Ingestion in the presence or absence of opsonin depended upon incubation time in the monolayer assay (data not shown). A comparison with *C. albicans* blastoconidia showed that non-opsonic ingestion did not occur (5.4 SD 4.4%).

Opsonic requirements for phagocytosis

Serum complement clearly promoted ingestion of arthroconidia to a significantly greater ($p < 0.005$)

Table I. Effect of arthroconidial concentration on neutrophil ingestion in the presence or absence of serum complement

Number of arthroconidia	Percentage (SD) ingestion*	
	without opsonin	with serum complement
5×10^4	18.3 (3.5)	41.0 (6.2)
1×10^5	23.1 (4.1)	44.4 (4.4)
3×10^5	31.9 (4.9)	40.0 (5.4)
5×10^5	24.9 (2.1)	59.2 (6.5)
7×10^5	26.7 (3.7)	56.4 (5.9)

*Percentage of neutrophils with ingested arthroconidia. All phagocytosis values represent the data for five individual neutrophil monolayers for each set of experimental conditions.

Table II. Role of anti-*T. mentagrophytes* serum as an opsonin

Opsonin	Percentage (SD) ingestion
None	19.3 (2.8)
Serum 10 %	61.1 (5.2)
Antiserum 10 %	40.5 (0.7)
Antiserum 10 % + antigen	25.7 (3.4)

Table III. Effect of exposing *T. mentagrophytes* arthroconidia to site-specific enzymes on neutrophil phagocytosis

Opsonin	Percentage (SD) ingestion after exposure to		
	trypsin	proteinase	chitinase
None	18.6 (4.0)	6.3 (1.5)	4.5 (3.5)
Serum	34.0 (1.4)	4.0 (1.0)	20.6 (5.1)

level than that seen for arthroconidia in the absence of opsonin (table II). Specific antiserum promoted ingestion (40 %), but when an antigenic extract of *T. mentagrophytes* was added to the antiserum before addition to the ingestion assay, the level of ingestion was reduced to a level not significantly different from that seen in the absence of an opsonin ($p > 0.1$).

Phagocytosis of arthroconidia exposed to enzymes

Exposure of arthroconidia to trypsin inhibited complement binding as expressed by a reduction in ingestion to 34 % (table III). Trypsin did not increase or decrease the level of non-opsonic ingestion. Proteinase treatment reduced ingestion to negligible levels. Chitinase had a similar effect but some complement binding occurred (20.6 % ingestion).

Blocking of mannose receptors on neutrophil membranes

In an attempt to determine the contribution of mannose-specific attachment in neutrophil ingestion of arthroconidia, D(+)mannose was added to glass-adherent neutrophils. The monolayers were washed

Table IV. Effect of adding mannose to human neutrophils prior to arthroconidia phagocytosis

Opsonin	Percentage (SD) ingestion
None	18.7 (3.4)
Serum	36.6 (4.9)

and arthroconidia were then added. Non-opsonic ingestion was not affected but the level of complement-dependent phagocytosis was significantly reduced from 60 % to 36.6 % ($p < 0.01$) (table IV).

Discussion

In dermatophytosis, effector mechanisms that contribute to the control of fungal growth in the epidermis are poorly understood. It is evident that infiltration of macrophages and neutrophils occurs before the peak of infection in experimental models.¹³ Although dermatophyte antigens are chemotactic *in vitro* for human neutrophils,^{14,15} the role of such cells in defence against dermatophytes has not been firmly established. Furthermore, Dahl and Carpenter⁶ showed that neutrophils adhered well to fungi provided the fungal hyphae had been pre-incubated with fresh human serum as a source of complement. These and other studies used saprophytic microconidia as target cells in various biological assays to assess phagocytosis and intracellular killing. The present study specifically examined the interaction between tissue phase arthroconidia of *T. mentagrophytes* and human neutrophils.

Generally, fungi are only phagocytosed after they have been opsonised. The role of opsonins in the binding of fungal cells such as blastoconidia of *C. albicans* and conidia of *Aspergillus fumigatus* is indisputable. Complement activation results in an accumulation of neutrophils but many areas of the body are poor in complement (e.g., lungs, cerebrospinal fluid and skin), and, because many micro-organisms interact with phagocytic cells in serum-free systems *in vitro*, there must be a number of mechanisms of non-opsonic phagocytosis. Some micro-organisms are able to adhere to neutrophils in the absence of antibody or complement. Adhesins are important components that mediate adherence of micro-organisms to neutrophils in the absence of opsonin. Based on bacterial systems, two major non-opsonic recognition mechanisms are known, and a considerable amount of information on the chemical nature of the complementary molecules on the bacteria and phagocytes is available. The more thoroughly studied mechanism, termed lectinophagocytosis, is based on recognition between surface lectins on one cell and surface carbohydrates on the adjacent cell. The other involves protein-protein interaction *via* the Arg-Gly-Asp peptide sequence.

The few studies on dermatophyte cell and human phagocyte interactions have focused on complement-

dependent systems. Indeed, the present study confirmed that maximal phagocytosis of arthroconidia is promoted by complement and specific antibody. These findings suggest that direct and specific recognition between molecules on the arthroconidial surface and complementary structures on the phagocyte surface exist as an alternative mechanism of phagocytosis.

Of direct relevance to the binding of neutrophils to dermatophyte conidia is the presence of unique resistant layers in the arthroconidial wall. In contrast to the saprophytic microconidia, *T. mentagrophytes* arthroconidia do not possess a rodlet layer on their surface.¹⁶ Instead, they have another type of resistant layer in the cell wall.¹⁷ The mature arthroconidia of *T. mentagrophytes* are surrounded by a thick inner wall, which is synthesised *de novo* during arthroconidogenesis, and by the outer wall, the remnant of the hyphal wall.¹⁸ Chemically, two-thirds of the isolated arthroconidial wall is chitinous in nature and comprised of two distinct forms of chitin: the chitinase-sensitive microfibrillar form and the chitinase-resistant non-fibrillar form.¹⁷ This unique chitin-rich layer may be responsible for the resistance of arthroconidia to antifungal agents. In the present study, both proteinase and chitinase reduced the level of non-opsonic phagocytosis of arthroconidia, suggesting that outer surface components of the arthroconidial cell wall are implicated in binding and phagocytosis in the absence of opsonin.

Lectins on the arthroconidial wall may facilitate non-opsonic phagocytosis. The data from the present study suggest that lectinophagocytosis is mediated by interaction of cell-wall lectins with mannose-containing glycoproteins on the phagocyte surface. Mannose was an effective inhibitor of phagocytosis when added to neutrophil monolayers in the presence of serum. This suggests that mannose receptors on the phagocyte membrane were partially blocked when arthroconidia were opsonised, but the level of non-opsonic binding

and ingestion was not reduced. Whether the mannose-specific attachment to phagocytes will lead to ingestion and killing appears to depend on the underlying surface properties of the fungal cell.

Other mechanisms of non-opsonic ingestion of dermatophyte arthroconidia are suggested by the present data but have yet to be explored. In a number of bacterial-effector cell interactions, surface hydrophobicity correlates with the ability of microbial cells to bind to phagocytic cells. It is possible, that surface hydrophobins serve to overcome repulsive forces between the fungal and phagocytic surfaces to allow more specific interactions to take place with higher avidity. An alternative suggestion is that a rough hydrophobic surface may facilitate phagocytosis, whereas a smooth hydrophilic one will counteract ingestion in the absence of an opsonin.

Another serum factor other than complement or antibody that functions as an opsonin is lipopolysaccharide binding protein. This protein is an acute-phase reactant that binds bacterial liposaccharide. Lipopolysaccharide binding protein can bind to the surface of gram-negative bacilli and strongly enhances attachment of these particles to the CD-14 molecule in the cell membrane of phagocytic cells. Whether such a mechanism promotes binding of dermatophyte arthroconidia to neutrophils remains speculative.

In the present study, opsonisation of the arthroconidia was not required to initiate phagocytosis, unlike the requirement for serum complement or specific antibody as opsonins for *C. albicans*. The results indicate that phagocytosis of dermatophyte arthroconidia relies on an alternative mechanism for the recognition of an invading micro-organism. Selective removal of cell-wall components before phagocytosis indicated direct and specific recognition between molecules on the microbial surface and complementary structures on the phagocyte surface.

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