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Fungicidal activity of human neutrophils and monocytes on dermatophyte fungi, *Trichophyton quinckeanum* and *Trichophyton rubrum*

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SUMMARY

Human peripheral polymorphonuclear neutrophils exhibited potent cytotoxic activity against the dermatophyte fungi *Tricophyton quinckeanum* and *T. rubrum* as assessed by inhibition of fungal replication in Sabouraud's agar. Monocytes also showed cytotoxic activity, but this was less pronounced than that of neutrophils, while lymphocytes had no toxic effect. Cytotoxicity showed a linear relationship to the target cell : effector cell ratio, with significant killing detected at a ratio of one neutrophil to one fungal cell. Fungal killing was optimal at incubation times of 2–24 hr for *T. rubrum* and 2–48 hr for *T. quinckeanum*. Thereafter, neutrophils were unable to prevent fungal replication while remaining viable. Cytotoxicity was markedly reduced by sodium azide, an agent that inhibits haem enzymes, and by catalase, but not by heat-inactivated catalase or superoxide dismutase. The fungicidal activity of neutrophils and monocytes was greatly increased by stimulation with phorbol myristate acetate (PMA) or with concanavalin A (Con A) compounds known to stimulate the secretion of lysosomal enzymes and the production of highly reactive oxygen intermediates. The cytotoxic activity of monocytes to *T. quinckeanum*, but not to *T. rubrum*, was also increased by Con A treatment. Neutrophil and monocyte phagocytosis of dermatophytes was demonstrated by electron microscopy studies. Disrupted *T. quinckeanum* and *T. rubrum* germlings were identified in the cytoplasm of the phagocytic cells, and similarly disruption of hyphae surrounded, but not engulfed, by neutrophils was also observed. These studies suggest that phagocytosis and/or oxidative products of the respiratory burst of neutrophils and monocytes may be implicated in the killing of dermatophytes *in vivo*.

INTRODUCTION

Human polymorphonuclear neutrophils and monocytes play an important role in host defence against bacteria, parasites, fungi and tumour cells (Klebanoff, 1975; Clark & Klebanoff, 1975). Phagocytosis of microbes by human neutrophils and monocytes is associated with dramatic changes of the oxidative metabolism. These metabolic events are referred to as the respiratory burst (Babior, 1978). The changes include an increase in oxygen consumption with subsequent production of highly toxic oxygen intermediates such as hydrogen peroxide, superoxide anion, hydroxyl radicals and singlet oxygen. A similar enhancement of the respiratory burst can be induced *in vitro* by stimulation of resting human neutrophils and monocytes with certain surface-active components, such as phorbol myristate acetate (PMA) (DeChatelet, Shirley & Johnson, 1976), and lectins (Simchowitz & Schur, 1976), including concanavalin A (Con A) and phytohaemagglutinin.

Dermatophyte infections are caused by septate mould fungi which invade the keratinized tissue of skin, hair and nails in humans and animals. The importance of neutrophils in the host defence against dermatophytes is suggested by a dense infiltration of neutrophils observed in infected areas of the skin in both naturally occurring animal ringworm in man (Roberts & Mackenzie, 1979) and experimentally infected mice (Hay, Calderon & Collins, 1983).

The oxidative system described by Klebanoff (1968), which includes myeloperoxidase, hydrogen peroxide and an oxidizable cofactor such as KI, or NaCl, is known to be effective in the killing of fungi (Lehrer, 1975). In a previous report we demonstrated that *T. quinckeanum* and *T. rubrum* were susceptible to killing by oxygen intermediates using a phagocyte-free system (Calderon & Shennan, 1987). In the present study, using an *in vitro* phagocytic system, we provide further evidence to suggest that phagocytic cells may play an important role in resistance to dermatophytosis.

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MATERIALS AND METHODS

Fungi

A previous report (Calderon & Shennan, 1987) described the

original source of isolates of *T. quinckeanum* and *T. rubrum* employed in this study, as well as their maintenance in culture and the preparation of germinated spores (germlings).

Isolation of neutrophils and mononuclear leucocytes
Heparinized (10 U/ml sodium heparin) blood from normal healthy volunteers was mixed with 6% Dextran 70 (Sigma Chemical Co., Poole, Dorset) in a ratio of 5:1 as described elsewhere (Mendelsohn, Skinner & Kornfeld, 1971). Briefly, blood was allowed to sediment at 37° for 45 min. The leucocyte-enriched plasma was removed and layered over Ficoll–Hypaque ($d = 1{\cdot}077$, Flow Laboratories Ltd, Irvine, Ayrshire) in a ratio of 2:1. Tubes were centrifuged at 1500 ***g*** for 10 min. Mononuclear cells were collected from the interface and washed twice with phosphate-buffered saline (PBS) 0·15 M, pH 7·2. These cells were further fractionated in a Percoll gradient (see below). In order to obtain the neutrophils, the pelleted cells were collected and the remaining erythrocytes lysed with 0·83% NH_4Cl in 0·01 M Tris–HCl, pH 7·4, for 5 min at 37°. Neutrophils were washed three times and resuspended in RPMI-1640 medium. Cell viability was estimated by dye exclusion using a solution of 0·2%(w/v) eosin in PBS.

Purification of monocytes and lymphocytes
Monocytes and lymphocytes from a mononuclear cell pool were separated on a discontinuous gradient of Percoll as described by Al-Sumidaie, Jones & Young (1984). Briefly, three iso-osmolar densities of Percoll, 1·057 g/ml, 1·066 g/ml and 1·074 g/ml, were prepared in Eagle's medium. Up to 4×10^6 mononuclear cells were resuspended in 2 ml of percoll density 1·074 g/ml in a 10-ml polycarbonate tube (Nunc, Roskilde, Denmark). A second layer (2 ml) of Percoll density 1·066 g/ml was gently dispensed over the first one, and finally a third layer of Percoll density 1·057 g/ml was dispensed on top of the second one. The tube was centrifuged at 2200 ***g*** for 90 min at room temperature. The monocytes found at the interface of the 1·057 g/ml and 1·066 g/ml densities, and the lymphocytes, found at the interface of the 1·066 g/ml and 1·074 g/ml densities, were carefully removed and washed three times with ice-cold RPMI-1640 medium. Viability was always >97% by eosin dye exclusion. Between 85% and 90% cells of the monocyte fraction were esterase-positive, as judged by the non-specific esterase staining method of Yam, Li & Crosby (1971).

Fungicidal activity of neutrophils and mononuclear leucocytes
Polymorphonuclear neutrophils, monocytes or lymphocytes were resuspended in RPMI-1640 medium (Gibco, Paisley, Renfrewshire) supplemented with 2 mM glutamine, 25 mM HEPES, penicillin/streptomycin (100 U/ml) (Gibco) and 5% autologous serum (AS) were mixed with germlings (5×10^4) of *T. quinckeanum*, or *T. rubrum* at cell ratios ranging from 1:1 to 100:1 in 10-ml conical tubes. The total volume was 1·0 ml. Control tubes contained 5×10^4 germlings without the leucocyte fractions. The germling–cell mixture was centrifuged at 25 ***g*** for 4 min immediately before incubation for 2 hr in a humidified atmosphere of 5% CO_2 air. In some experiments cells were incubated for 1–3 days. After incubation cells were washed three times and resuspended in phosphate-buffered saline (PBS) 0·15 M, pH 7·2. Cells were vigorously shaken in a Vortex and then passed through a syringe to disrupt any clumps. A sample of the mixture containing approximately 250–300 germlings was mixed with liquid neutral glucose peptone (NGP)–1·5% agar and plated out in small (5 mm diameter) petri-dishes. Assays were done in triplicate tubes, and for each tube three petri-dishes were inoculated. Petri-dishes were incubated at 28° for 3–6 days, and colony-forming units (CFU) per plate were counted against an illuminated background. A control experiment comparing the viability of phagocyte/germling mixtures with and without disruption of host cells using distilled water containing 0·01% albumin showed no significant difference in resulting colony counts. Percentage dermatophyte viability was calculated as:

$$\%\ \text{viability} = \frac{\text{CFU in test culture}}{\text{CFU in control}} \times 100.$$

Activation of cells with Con A and PMA
In additional experiments, neutrophils, monocytes and lymphocytes were stimulated to produce oxygen metabolites by treatment with PMA or Con A (3 × cryst; Miles Yeda Ltd, Slough, Berks). Phorbol myristate acetate (Sigma) was dissolved in dimethyl sulphoxide to give a stock solution of 2 mg/ml, and stored at −20°. The stock solution was diluted in PBS immediately before use. Con A was prepared in PBS and stored at −20° at a concentration of 10 mg/ml. Human leucocytes were incubated with *T. rubrum* or *T. quinckeanum* germlings for 15 min before the addition of either PMA (10 ng/ml)) or Con A (100 μg/ml).

Electron microscopy
Monocytes (5×10^6) or neutrophils (5×10^6) were incubated with germlings (5×10^6) of *T. quinckeanum* or *T. rubrum* as described above. After incubation, cells were fixed in 3% glutaraldehyde in cacodylate buffer, 0·066 M, pH 7·4, containing 0·01% $CaCl_2$, for 1 hr. Cells were then resuspended in 2% noble agar and washed overnight in cacodylate buffer containing 0·2 M sucrose. Specimens were fixed in 1% osmium tetroxide for 90 min, washed in distilled water, then placed in 30% methanol. Specimens were stained with 2% uranyl acetate in 30% methanol for 25 min before dehydration in graded methanols. They were embedded in TAAB resin. Ultrathin sections were cut on Huxley MK II grids and stained in Reynolds' lead citrate (Reynolds, 1963). Sections were examined with a JEOL 100 cx EM at 80 kV.

Special reagents
Percoll (Pharmacia Fine Chemicals, Milton Keynes, Bucks) was prepared by mixing nine parts of Percoll density 1·13 g/ml with one part of 10 times strength Eagle's medium (Wellcome Research Labs, Beckenham, Kent). Superoxide dismutase (2900 U/mg protein) and catalase from bovine liver (25,000 U/mg protein) were obtained from Sigma. Catalase was heat-inactivated at 100° for 15 min. Sodium azide was purchased from BDH Chemicals Poole, Dorset. Enzymes and inhibitor were incubated with the effector and target cells for 15 min before the addition of Con A or PMA.

Statistics
Standard error was employed as an estimate of variance, and means were compared by Student's two-tailed *t*-test.

RESULTS

Cytotoxic effect of neutrophils on fungi

Germlings (5×10^4) of *T. quinckeanum* or *T. rubrum* alone, or

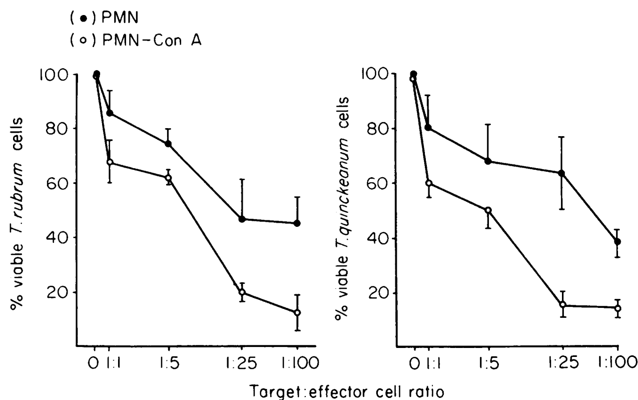

Figure 1. Effect of neutrophil, and Con A-stimulated neutrophil numbers on toxicity of dermatophytes. Germlings (5×10^4) of *T. rubrum* or *T. quinckeanum* were mixed with neutrophils in RPMI-1640 medium with 5% autologous serum, in the presence or absence of 100 μg Con A. The total volume was 1·0 ml. The germling–neutrophil cell mixture was incubated for 2 hr at 37° in a CO_2 atmosphere. In control samples fungal cells were incubated alone, or in the presence of Con A. Values are the percentage of control samples and represent the arithmetic mean (±SE) of five separate experiments. The values between unstimulated and Con A-stimulated neutrophils are significant, with $P < 0{\cdot}001$ at all cell ratios.

combined with different numbers of neutrophils, were incubated in RPMI medium containing 5% autologous serum for 2 hr at 37° in a CO_2 atmosphere. A high cytotoxic activity judged by a marked reduction in cell replication of both species of dermatophytes in Sabouraud's (NGP) medium was observed (Fig. 1). Some cytotoxicity occurred at a target:effector cell ratio of 1:1 ($P < 0{\cdot}01$), but there was more significant toxicity at ratios of 1:5 ($P < 0{\cdot}001$). The cytotoxicity increased gradually with the number of effector cells. For *T. rubrum* cytotoxicity reached a maximum at ratios of one germling to 25 neutrophils, whereas for *T. quinckeanum* maximal cytotoxicity was observed at a ratio of 1:100. In additional experiments where neutrophils and fungus were incubated for 2 hr in RPMI medium containing 0·5% autologous serum (AS), the cytotoxic activity was comparable to that observed with 5% AS (data not shown). Furthermore, in experiments where leucocytes and fungus were incubated for 2 hr in 0·03 M sodium phosphate buffer, pH 7·0, $1{\cdot}5 \times 10^{-3}$ M KH_2PO_4, $1{\cdot}5 \times 10^{-3}$ M $MgSO_4$ containing 0·25% AS, there was only a slight reduction in cytotoxic activity. For instance, under these conditions the killing of *T. quinckeanum* at target to effector cell ratios of 1:25 was 74% ± 7 as opposed to 64% ± 12 using RPMI–5% AS medium. Autologous serum (5%) alone in RPMI not only had no toxic effect on the fungus, but enhanced its viability in the culture conditions employed here. For example, when 200 germlings of *T. quinckeanum* incubated in RPMI medium without serum were plated onto NGP–agar plates, only 45% ± 5 CFU appeared, whereas in RPMI medium containing 5% AS an average of 92% ± 7 CFU were counted. For these experiments samples from 10 healthy volunteers were employed.

The cytotoxic activity of neutrophils on dermatophytes was further increased by stimulation with Con A, an agent known to induce formation of oxidative intermediates. Neutrophils were incubated with fungi in RPMI medium–5% AS for 2 hr in the presence of 100 μg/ml Con A. Both dermatophyte species showed a slight susceptibility to Con A alone; a 5–15% killing of fungal spores was regularly obtained. Therefore, experimental groups containing Con A were compared against a control Con A-treated fungus. Results are shown in Fig. 1. Enhanced cytotoxicity was induced by stimulation of neutrophils with Con A. Here, 30–40% killing ($P < 0{\cdot}001$) was observed at a target to effector cell ratio of 1:1, and up to 90% killing at ratios of 1:25 to 1:100.

Effect of time in culture on cytotoxic activity of neutrophils and monocytes

The time–course of dermatophyte killing by neutrophils and monocytes was determined. Neutrophils or mononuclear leucocytes (5×10^4) were incubated with *T. quinckeanum* or *T. rubrum* germlings ($1{\cdot}25 \times 10^6$) at 37° in medium containing 5% autologous serum at time intervals of 2, 24, 48 or 72 hr. A substantial neutrophil cytotoxicity was shown at incubation times of 2–48 hr for *T. quinckeanum* (60% killing), and at 2–24 h for *T. rubrum* (50–70% killing) (Fig. 2). However, an unexpected enhancement in viability for *T. quinckeanum* and *T. rubrum* was observed after 48 hr or 24 hr of incubation, respectively. Viability of the neutrophils remained above 60% throughout the 72 hr incubation period as judged by the eosin dye exclusion test.

Mononuclear leucocytes, on the other hand, showed a less pronounced (12–20%) cytotoxic activity for both fungal species at incubation times between 2 hr and 48 hr, and only a modest enhancement in viability at 72 hr (see Fig. 2).

Effect of Con A and PMA on the activity of neutrophils, monocytes and lymphocytes

Polymorphonuclear neutrophils and fractions of monocytes and lymphocytes separated on a discontinuous gradient of Percoll were used to investigate the activity of these cells on dermatophytes in the presence of phorbol myristate acetate, or

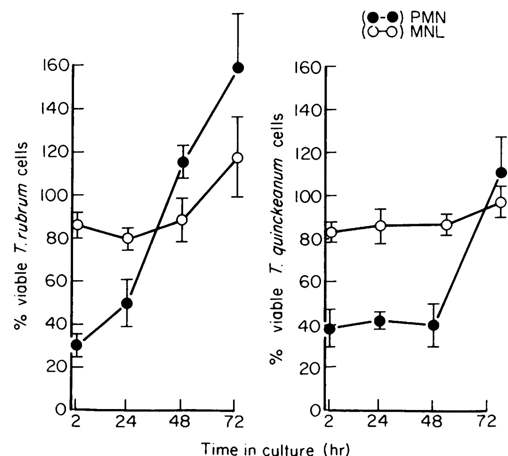

Figure 2. Time–course effect of neutrophil and mononuclear leucocytes on dermatophyte viability. Phagocytic cells ($1{\cdot}25 \times 10^6$) were mixed with 5×10^4 *T. rubrum*, or *T. quinckeanum* germlings as described in Fig. 1. The incubation times were varied as indicated. Values represent the arithmetic mean (±SE) of five experiments.

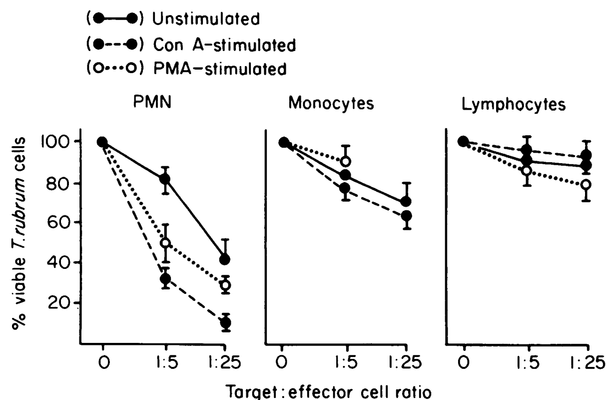

Figure 3. Effect of Con A- or PMA-stimulated neutrophils, monocytes and lymphocytes on *T. rubrum* viability. Germlings (5×10^4) of fungus were incubated for 2 hr with unstimulated, Con A- (100 μg/ml) stimulated, or PMA- (10 ng) sitmulated leucocytes in the proportions indicated. Monocytes and lymphocytes were purified in a discontinuous gradient of Percoll (see Materials and Methods). Values represent the arithmetic mean (±SE) of six separate experiments. $P < 0.001$ for Con A-stimulated neutrophils at 1:5 and 1:100 target:effector cell ratios and for PMA-stimulated at 1:5 ratio, compared to unstimulated cells. There is no significant difference between stimulated and non-stimulated monocytes or lymphocytes.

Con A. Germlings (5×10^4) of *T. rubrum* or *T. quinckeanum* were incubated with 25×10^4 or $1{\cdot}25 \times 10^6$ neutrophils, monocytes or lymphocytes in the presence of PMA (10 ng/ml) or Con A (100 μg/ml) for 2 hr at 37°. Figure 3 shows the results with *T. rubrum*. The cytotoxic activity of neutrophils on *T. rubrum* was significantly increased after stimulation with PMA or Con A at a target:effector cell ratio of 1:5 ($P < 0{\cdot}001$). On the other hand, there was only a marginal increase in the cytotoxicity of unstimulated *vs* Con A-stimulated monocytes at ratios of 1:5 and 1:25 from 16–30% killing to 23–37% killing, respectively, and the lymphocyte fraction showed negligible killing. Results for *T. quinckeanum* tested at target:effector cell numbers of 5×10^4:$1{\cdot}25 \times 10^6$ are shown in Table 1. Similar results to those of *T. rubrum* were obtained with *T. quinckeanum*, except for monocytes which showed a substantial increase in fungicidal activity after Con A stimulation from 18% killing to 38% killing ($P < 0{\cdot}001$). Moreover a mixture of neutrophils ($6{\cdot}25 \times 10^5$) and monocytes ($6{\cdot}25 \times 10^5$) failed to have an enhancement or inhibitory effect in the cytotoxicity assay compared to neutrophils alone. The apparent reduction in the cytotoxic activity of neutrophils may be due to the lower number of cells.

Table 1. Effect of human polymorphonuclear neutrophils, monocytes and lymphocytes on *T. quinckeanum* viability*

Cell source	*T. quinckeanum*	Con A (100 μg/ml)	CFU per plate†	Viability (% control)
None (control)	+	−	216 ± 18	100
PMN	+	−	115 ± 19	53‡
Monocytes	+	−	177 ± 17	82‡
Lymphocytes	+	−	205 ± 20	95
None (control)	+	+	194 ± 11	100
PMN	+	+	37 ± 16	19‡
Monocytes	+	+	120 ± 21	62‡
PMN + monocytes	+	+	68 ± 12	35‡
Lymphocytes	+	+	182 ± 13	94

* *T. quinckeanum* germlings (5×10^4) were incubated for 2 hr with various human leucocyte fractions ($1{\cdot}25 \times 10^6$) in the presence or absence of concanavalin A.

† Values represent the arithmetic mean ± SEM of three separate experiments. Each experimental group was run in triplicate. Statistical analysis comparing test *vs* control: ‡$P < 0{\cdot}001$; § $P < 0{\cdot}005$.

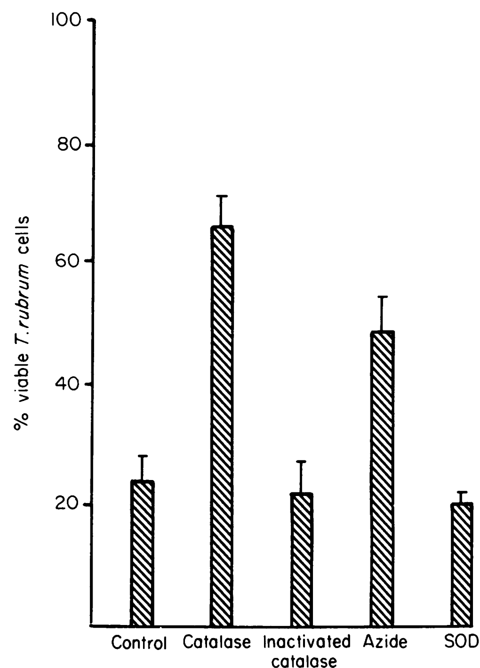

Figure 4. Effect of catalase, sodium azide and SOD on the Con A-stimulated neutrophil-mediated killing of *T. rubrum*. The assay conditions are the same as those described in Fig. 1 using a 1:25 target:effector cell ratio. Catalase (290 U/ml), heat-inactivated (100°, 15 min) catalase, sodium azide (0·1 mM) and superoxide dismutase (2500 U/ml) were preincubated for 15 min with the effector cells before the addition of Con A. Results are expressed as the mean percentage of control viability of four experiments. $P < 0{\cdot}001$ for catalase- and azide-treated neutrophils, compared to control.

Effect of catalase, sodium azide and superoxide dismutase on neutrophil cytotoxicity

The role of oxidative intermediates on the cytotoxic activity of neutrophils was evaluated using scavengers and inhibitors of various oxygen metabolites such as catalase (H_2O_2 scavenger), superoxide dismutase (SOD) (superoxide anion) and sodium azide (myeloperoxide inhibitor). Neutrophils ($1{\cdot}25 \times 10^6$) and *T. quinckeanum* germlings (5×10^4) were incubated with catalase (290 U/ml), heat-inactivated (100°, 15 min) catalase, sodium azide (0·1 mM), or SOD (2500 U/ml) in RPMI–5% AS for 2 hr at 37°. Con A (100 μg/ml) was added 15 min after enzymes and inhibitor. Figure 4 shows the results. A significant inhibition of neutrophil cytotoxicity to *T. quinckeanum* from 76%to 32% killing was observed in the presence of catalase. Heat-inactivated catalase, however, had no effect on cytotoxicity. The Con A-induced neutrophil cytotoxicity was also reduced by sodium azide, but not by superoxide dismutase. Neither catalase, SOD, nor sodium azide alone had a toxic effect on the fungus. Thus, the ability of catalase and sodium azide to inhibit neutrophil

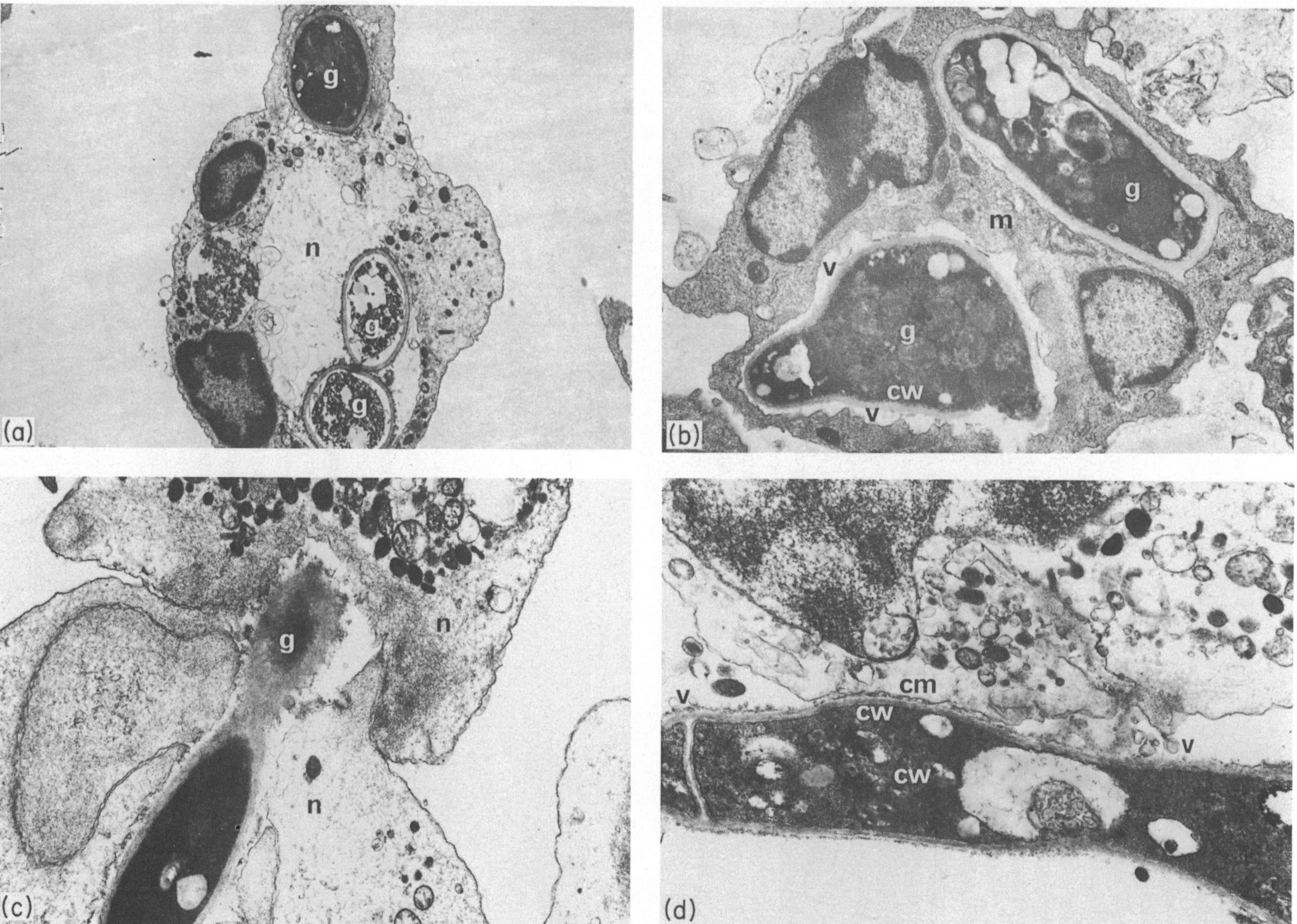

Figure 5. Electromicrographs of phagocyte–germling interaction. (a) *T. rubrum* germlings within a neutrophil (n). Varying degrees of cytoplasmic disorganization are seen with the germlings (g) (magnification × 6000). (b) *T. quinckeanum* germlings (g) within a monocyte (m). The fungal cell wall (cw) is indistinct and thick. Vesicle-like structures (v) are seen (magnification × 9000). (c) Germling (g) of *T. rubrum* surrounded by two neutrophils (n) magnification × 12,000). (d) Hyphal germling (g) of *T. rubrum* attached to the exterior of a neutrophil. There is a close apposition between the phagocyte cell membrane (cm) and the fungal cell wall (cw). Note the presence of vesicle-like structures (v) (magnification × 12,000).

cytotoxicity suggests that the H_2O_2–halide–myeloperoxidase system may play a crucial role in the fungicidal activity.

Ultrastructural studies of phagocyte–germling interaction

Electron micrographs were prepared from cocultures of Con A-stimulated neutrophils, or monocytes and germlings of *T. quinckeanum* or *T. rubrum*, mixed in a ratio of 1:25 fungal to effector cells. The fungal–phagocytic cell mixture was preincubated for 2 hr at 37° in RPMI–5% AS. Results are shown in Fig. 5. Human neutrophils were found to ingest germlings and conidia readily with up to six intact fungal elements per cell. In most cases organisms were clearly seen to be surrounded by a host cell membrane and appeared to be within a phagosome. Intracellular disruption was the most noticeable change associated with thickening of and loss of definition in the fungal cell wall. There was no difference in the appearance of the interaction between host cells and either *T. quinckeanum* or *T. rubrum*. However, it was noticed that in some cases hyphal elongation had occurred and it was not possible for the neutrophil to engulf the organism. In this case a second neutrophil was sometimes found to engulf or surround the protruding hypha. In cases where hyphae were not ingested, close apposition between macrophage cell membrane and fungal cell wall was observed and disruption of the fungus found to have occurred. This suggests that both intracellular and extracellular killing mechanisms may occur in this system. In both examples small vesicular structures could be seen between the phagosome or macrophage cell membrane and the fungal cell wall. The significance of these structures is unknown, but they may be involved in the killing process.

Human monocytes show similar avidity for germlings of both species, and once again fungal cells are taken up and show internal disruption.

DISCUSSION

The present study examined the fungicidal activity of human neutrophils and monocytes against the two species of dermatophyte, *T. quinckeanum* and *T. rubrum*. The anthropophilic species *T. rubrum* is one of the most common causes of dermatophytosis in humans, accounting for approximately 80–90% of chronic human cases (Hay, 1982). *T. quinckeanum* is a zoophilic species which produces a characteristic infection, favus, in mice that can be reproduced experimentally (Hay *et al.*, 1983). The results of this study suggest that neutrophils and monocytes may play an important role in resistance to dermatophytosis. Human neutrophils were highly toxic to germlings of both species of dermatophyte after 2 hr of incubation at 37°. Cytotoxicity showed a linear target to effector cell relationship with killing of 40–50% at a ratio of 1:25 cells (Fig. 1). Mononuclear leucocytes exerted a less marked, but nonetheless significant, cytotoxic effect; 20% killing was regularly observed 2 hr after incubation (Fig. 2). The cytotoxic activity of neutrophils and monocytes was transient, lasting for 24–48 hr of incubation. Longer periods of incubation resulted in increased fungal viability (Fig. 2). Although growth by these organisms is optimal at 25–30°, it was observed that fungal cell counts remained constant for at least 48 hr at 37° in RPMI medium containing 5% autologous serum. However, cell numbers were greatly increased when dermatophytes were incubated only with RPMI medium that had been adjusted to a slightly acidified pH ranging from pH 6·2 to 6·7 (data not shown). Neutrophils and monocytes in culture caused acidification of the RPMI medium, which increased with time. Therefore, it is likely that once the cytotoxic capacity of the phagocytic cells had been exhausted, the residual viable fungi would not simply just remain static, but would actually replicate in a favourable environment. Thus, the enhancement of fungal cell viability in the late phase of coculture with phagocytic cells depends on environmental conditions, and its relevance to an *in vivo* situation is dubious.

Using neutrophils and purified fractions of monocytes and lymphocytes separated in a discontinuous gradient of Percoll, the effect of agents such as Con A and phorbol myristate acetate (PMA), which stimulate the release of oxygen intermediates, was studied. The cytotoxic activity of neutrophils was significantly increased by the addition of Con A (Fig. 1). At a target:effector cell ratio of 1:25 an average cytotoxic index of 40–55% with unstimulated neutrophils was regularly obtained. This fungicidal activity was increased to 80–90% killing after stimulation with Con A. Likewise, PMA produced an increase in the cytotoxic activity of neutrophils (Fig. 3). Both Con A and PMA failed to increase killing of *T. rubrum* by monocytes, whereas their activity against *T. quinckeanum* was enhanced by Con A (Table 1). Neutrophils combined with monocytes failed to produce either enhancement or inhibition of the killer effect. Moreover, lymphocytes had no effect on the viability of either dermatophyte species (Fig. 3 and Table 1).

Although both neutrophils and monocytes share a number of functional activities such as phagocytosis, mobility within tissues to reach the site of an infection and the capacity to kill microorganisms intracellularly (Sbarra & Karnovsky, 1959), there are also differences between them. For instance, neutrophils release larger amounts of oxidative products of the respiratory burst, including superoxide anion, hydrogen peroxide and myeloperoxidase, than monocytes (Ross & Joke, 1980). Two observations support the possibility that products of the respiratory burst of neutrophils play an important role in the killing of dermatophytes. First the fungicidal activity of neutrophils was drastically reduced by the addition of catalase, an enzyme that catalyses the conversion of hydrogen peroxide into oxygen and water. Second, sodium azide, an inhibitor of myeloperoxidase, a haem enzyme, also reduced the toxic activity of neutrophils (Fig. 4). Since neutrophils possess a more active respiratory burst, it is not surprising that these cells have a more powerful anti-fungal activity than monocytes even though they appear to be equally active in phagocytosis. These results support our previous study in which we demonstrated that dermatophytes were susceptible to H_2O_2–peroxidase and halide ions using a phagocyte-free system (Calderon & Shennan, 1987). The involvement of oxidative mechansims in resistance to fungi other than dermatophytes has been reported previously. For instance, it has been shown that neutrophils from patients with chronic granulomatous disease, a condition in which neutrophils are unable to generate normal amounts of hydrogen peroxide, failed to damage *Candida* (Lehrer & Cline, 1969) and *Aspergillus* (Cohen *et al.*, 1981) hyphae. Products of the respiratory burst have the added advantage that they can participate in both the intracellular (Sbarra & Karnovsky, 1959; Ross & Joke, 1980) and extracellular (Nathon *et al.*, 1979) killing of microorganisms. However, the present study does not rule out the participation of other non-oxidative mechanisms in

the killing of dermatophytes by phagocytic cells. For instance, it has been reported that secretion of lysosomal enzymes from neutrophils and macrophages can be induced *in vitro* by stimulation with PMA, or Con A, or by induced phagocytosis (Goldstein, Hoffstein & Weissman, 1975). Macrophages also secrete neutral proteinases such as plasminogen activator (Unkeless, Gordon & Reich, 1974), collagenase and elastase (Werb & Gordon, 1975a, b), which are likely to participate in the cytotoxic activity.

The electron microscopic findings suggest that both neutrophils and monocytes can ingest and destroy *T. quinckeanum* and *T. rubrum* spores, and that with the former, and possibly the latter, extracellular killing of hyphae also occurred. Neutrophils in particular bind closely to hyphal forms, resulting in a cluster of phagocytes around a single fungal element. It is tempting to ascribe a secretory function to the small vesicles seen within phagosomes or adjacent to entrapped hyphae. However, the contents and significance of these structures are as yet unknown. Dermatophyte infections are characterized by invasion of the skin by hyphae. These hyphae are large and difficult to phagocytose *in situ*, thus, cytotoxicity by neutrophils should logically not depend solely on ingestion of the fungus. The importance of both intracellular and extracellular killing mechanisms in defence against other fungi such as *Candida albicans* has been emphasized by other investigators (Diamond, Krzesicki & Jao, 1978).

We conclude therefore that human neutrophils and, to a lesser degree, monocytes are potentially implicated in the host-defence mechanism against dermatophytes. This activity is largely mediated by oxidative intermediates derived from the respiratory burst of the phagocytic cells. Previously attention has been focused on the role of T lymphocytes in host defence against humans (Petrini & Kaaman, 1981) and experimentally infected animals (Calderon & Hay, 1984). However, from the studies reported above it appears that further work should be undertaken on the role of phagocytic cells and their relevance to the control of these infections.

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REFERENCES

AL-SUMIDAIE A.M., JONES D.L. & YOUNG H.L. (1984) Characterisation of the under-agarose method for quantifying migration of highly purified human monocytes. *J. immunol. Meth.* **75,** 129.

BABIOR B.M. (1978) Oxygen-dependent microbial killing by phagocytes. *New Eng. J. Med.* **298,** 659 and 721.

CALDERON R.A. & HAY R.J. (1984) Cell-mediated immunity in experimental murine dermatophytosis. II. Adoptive transfer of immunity to dermatophyte infection by lymphoid cells from donors with acute or chronic infections. *Immunology*, **53,** 465.

CALDERON R.A. & SHENNAN G.I. (1987) Susceptibility of *Trichophyton quinckeanum* and *Trichophyton rubrum* to products of oxidative metabolism. *Immunology*, **61,** 283.

CLARK R.A. & KLEBANOFF S.J. (1975) Neutrophil-mediated tumor cell cytotoxicity: role of the peroxidase system. *J. exp. Med.* **141,** 1442.

COHEN M.S., ISTURIZ R.E., MALECH H.L., ROOT R.K., WILFERT C.M., GUTMAN L. & BUCKLEY R.H. (1981) Fungal infection in chronic granulomatous disease: the importance of the phagocyte in defence against fungi. *Am. J. Med.* **71,** 59.

DECHATELET L.R., SHIRLEY P.S. & JOHNSTON R.B. (1976) Effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. *Blood*, **47,** 545.

DIAMOND R.D., KRZESICKI R. & JAO V. (1978) Damage to pseudohyphal forms of *Candida albicans* by neutrophils in the absence of serum *in vitro*. *J. clin. Invest.* **61,** 349.

GOLDSTEIN I.M., HOFFSTEIN S.T. & WEISSMAN G. (1975) Mechanisms of lysosomal enzyme release from human polymorphonuclear leukocytes: effect of phorbol myristate acetate. *J. Cell Biol.* **66,** 647.

HAY R.J. (1982) Chronic dermatophyte infections. I. Clinical and mycological features. *Br. J. Dermatol.* **106,** 1.

HAY R.J., CALDERON R.A. & COLLINS M.J. (1983) Experimental dermatophytosis: the clinical and histopathologic features of a mouse model using *Trichophyton quinckeanum* (mouse favus). *J. invest. Dermatol.* **81,** 270.

KLEBANOFF S.J. (1968). Myeloperoxidase–halide–hydrogen peroxide antibacterial system. *J. Bacteriol.* **95,** 2131.

KLEBANOFF S.J. (1975) Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Sem. Haematol.* **12,** 117.

LEHRER R.I. (1975) The fungicidal mechanisms of human monocytes. I. Evidence for myeloperoxidase-linked and myeloperoxidase independent candidacidal mechanisms. *J. clin. Invest.* **55,** 338.

LEHRER R.I. & CLINE M.J. (1969) Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to *Candida* infection. *J. clin. Invest.* **48,** 1478.

MENDELSOHN J., SKINNER S.A. & KORNFELD S. (1971) The rapid induction by phytohaemmagglutinin of increased α-aminoisobutyric acid uptake by lymphocytes. *J. clin Invest.* **50,** 818.

NATHAN C.F., SILVERSTEIN S.C., BRUKNER L.H. & COHN Z.A. (1979) Extracellular cytolysis by activated macrophages and granulocytes. II. Hydrogen peroxide as a mediator of cytotoxicity. *J. exp. Med.* **149,** 100.

PETRINI B. & KAAMAN T. (1981) T-lymphocyte subpopulations in patients with chronic dermatophytosis. *Int. Arch. Allergy appl. Immunol.* **66,** 105.

REYNOLDS E. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17,** 208.

ROBERTS S.O.B. & MACKENZIE D.W.R. (1979) Mycology. In: *Textbook of Dermatology* (eds A. J. Rook, D. S. Wilkinson and F. J. G. Ebling), 3rd edn, Vol. 1, p. 767. Blackwell Scientific, Oxford.

ROSS D. & JOKE S. (1980) The oxygen metabolism of human blood monocytes and neutrophils. In: *Mononuclear Phagocytes* (ed. R. V. Furth), Part II, p. 1219. Martinus Nijhoff Publishers, The Hague.

SBARRA A.J. & KARNOVSKY, M.L. (1959) The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J. biol. Chem.* **243,** 1355.

Simchowitz L. & Schur P.H. (1976) Lectin-dependent neutrophil mediated cytotoxicity. I. Characteristics. *Immunology*, **31,** 303.

UNKELESS J.C., GORDON S. & REICH E. (1974) Secretion of plasminogen activator by stimulated macrophages. *J. exp. Med.* **139,** 8348.

WERB Z. & GORDON S. (1975a) Secretion of a specific collagenase by stimulated macrophages. *J. exp. Med.* **142,** 346.

Werb Z. & Gordon S. (1975b) Elastase secretion by stimulated macrophages. Characterization and regulation. *J. exp. Med.* **142,** 361.

YAM L.T., LI C.Y. & CROSBY W.H. (1971) Cytochemical identification of monocytes and granulocytes. *Am. J. clin. Pathol.* **55,** 283.