Pneumocystis carinii-induced activation of the respiratory burst in human monocytes and macrophages

A. L. LAURSEN, B. MØLLER*, J. RUNGBY†, C. M. PETERSEN‡ & P. L. ANDERSEN *Department of Medicine and Infectious Diseases, Marselisborg Hospital, *Department of Clinical Immunology, Skejby Sygehus, †Department of Neurobiology, Institute of Anatomy, and Department of Endocrinology and Metabolism, Aarhus County Hospital, and ‡Department of Medical Biochemistry, University of Aarhus, Aarhus, Denmark*

SUMMARY

Human monocytes and monocyte-derived macrophages were studied for their ability to phagocytose *Pneumocystis carinii* and produce superoxide (O_2^-) during the process. One $\times$ 10^6 freshly isolated monocytes, incubated with $0.1–3.75 \times 10^6$ *P. carinii* cysts, increased O_2^- production in a dose-related way. Antibodies were essential for the process since opsonized, but not unopsonized, pneumocysts induced O_2^- production significantly above the response obtained by lung tissue from rats (10·7 and 4·9 *versus* 3·0 fmol/cell per 90 min). The difference between pneumocysts opsonized in untreated *versus* complement-depleted serum was not significant (10·7 *versus* 12·6 fmol/cell per 90 min). Monocyte-derived macrophages also activated the respiratory burst when stimulated with pneumocysts, and this effect could be significantly increased, from 4·2 to 8·8 fmol/cell per 90 min, when cells were primed with interferon-gamma (IFN-γ). Cells primed with IL-3 also increased O_2^- production, though to a lesser extent. In contrast, granulocyte-macrophage colony-stimulating factor (GM-CSF) had only a small effect on the respiratory burst in cells stimulated with *P. carinii*. Priming with IFN-γ increased the rate of phagocytosis in macrophages. After incubation for 90 min or more, however, the percentage of cells with phagocytic vacuoles was only slightly higher in IFN-γ-primed cells. When examined by electron microscopy (EM), most vacuoles contained partially or totally degraded pneumocysts. In conclusion, we have demonstrated the ability of monocytes and monocyte-derived macrophages to ingest and degrade pneumocysts, activating the respiratory burst during the process.

Keywords *Pneumocystis carinii* superoxide phagocytosis interferon-gamma IL-3

INTRODUCTION

Pneumocystis carinii, a eukaryote microorganism of uncertain taxonomic classification, but probably a fungus, is a well known cause of pneumonia in immunosuppressed patients. Congenital disorders, such as severe combined immunodeficiency and hypogammaglobulinaemia [1,2], as well as acquired conditions, such as malignancies and especially infection with HIV, may lead to *P. carinii* pneumonia (PCP). The infection is seen in HIV-infected patients with a low number of $CD4^+$ cells [3], but also in patients with hypogammaglobulinaemia. Although a T cell defect has been described in some patients in the latter group, the most prominent finding here is a low production of antibodies. In addition, infusion of MoAbs against *P. carinii* in immuno-

Correspondence: Alex L. Laursen, Department of Medicine and Infectious Diseases, Marselisborg Hospital, University of Aarhus, P. P. Ørumsgade 11, DK-8000 Aarhus C, Denmark.

suppressed animals has also given some protection against PCP [4], and this suggests that antibodies may play a role in the defence against *P. carinii* infections. Thus, both cellular and humoral immunity seem important for resistance against PCP. Antibodies are likely to act in conjunction with phagocytic cells. The respiratory burst, characterized by production of superoxide ions (O_2^-), is known to be important for the ability of phagocytic cells to kill ingested microorganisms. Previous studies, using chemoluminescence or peroxidase assay for H_2O_2 determination, showed that pneumocysts were able to activate the respiratory burst in rat macrophages and a macrophage cell line [5,6]. We have studied the ability of human monocytes and monocyte-derived macrophages to produce O_2^-, when stimulated with *P. carinii*. The importance of opsonization and the effect of interferon-gamma (IFN-γ) for this process were also investigated, since the production of this cytokine is impaired in AIDS patients [7,8]. The effect of two other cytokines, granulocyte-macrophage colony-stimulating

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factor (GM-CSF) and IL-3, was also studied, because they have previously been shown to prime macrophages to increased biocidal activity against other microorganisms [9,10].

MATERIALS AND METHODS

Production of Pneumocystis carinii
Parasites were produced and purified essentially as described by Walzer et al. [11]. Wistar male rats weighing 200–250 g were immunosuppressed with dexamethasone 1 mg/l, and tetracycline 1 mg/ml was added to the drinking water. Low protein diet was given.

When signs of pneumonia developed, usually after 6–8 weeks, the rats were killed, and the lungs aseptically removed. Imprints from each lung were stained by Giemsa and methenamine silver. Lungs with bacterial or non-pneumocystis fungal infections were discarded, and only those with moderate to severe *P. carinii* infections were used for preparation of pneumocysts (imprints showing more than 50 *P. carinii* cysts/field at magnification ×1000).

Lung tissue was cut into small pieces and transferred to a vial containing 10 mM sodium citrate in PBS. The mixture was agitated on a magnetic stirrer for 30 min at room temperature, the suspension was then filtered through sterile gauze and pelleted at 11000 g for 15 min. The pellet was digested with 0·2% collagenase (Sigma, St Louis, MO), 0·2% hyaluronidase (Sigma), and 0·4% deoxyribonuclease (Sigma) for 60 min at 37°C and subsequently centrifuged on a continuous Percoll gradient (Pharmacia, Uppsala, Sweden) for 30 min at 31000 g. Layers with densities from 1·018–1·062 g/ml contained a high number of pneumocysts. After three washes in PBS (11000 g, 15 min) the number of cysts was estimated and the suspension stored at −70°C until use.

Enumeration of cysts
To determine the number of *P. carinii* cysts, 10 μl of the solution were diluted and spread circularly on a slide, air dried, and fixed in methanol. The slides were stained with methenamine silver to identify cysts, counted, and the number of cysts/ml estimated.

Lung control tissue
Lungs from six healthy male rats, not exposed to dexamethasone, were aseptically removed, cut into small pieces, frozen in liquid nitrogen, and crushed with mortar and pestle. The tissue was filtered through gauze and treated with enzymes as described above. The protein content was measured [12]. A pneumocyst suspension was treated similarly, and control tissue was adjusted to the same protein concentration as the pneumocyst preparation. Lung control tissue is referred to as tissue control.

Opsonization
Pneumocysts and tissue control were trypsinized at 37°C for 30 min using 0·25% trypsin (Sigma type XI; 6630 BAEE units/mg protein) to remove bound antibodies and protein covering the parasites, washed in PBS, resuspended in 70% pooled blood group AB donor serum from two donors, and incubated at 37°C for 30 min. Pneumocysts treated in this way were examined by indirect immunofluorescence for the presence of rat antibodies using rabbit anti-rat immunoglobulin (Dakopatts, Copenhagen, Denmark) with an FITC/protein

ratio (F/P) of 2·3. No antibody could be detected. When indicated, serum was pretreated with 10 mM EDTA to remove calcium and magnesium in order to abolish complement reaction. The presence of IgG and complement on pneumocysts opsonized with untreated serum was determined by immunofluorescence. FITC-conjugated rabbit anti-human IgG and anti-C3c (Dakopatts), both with an F/P of 2·3, were used.

Isolation and cultivation of monocytes
Human peripheral blood mononuclear cells (PBMC) were isolated from citrate-phosphate-dextrose-stabilized buffy coats by flotation on Isopaque–Ficoll (Nyegaard, Oslo, Norway). Before flotation, 5 ml of 0·1% adenosine 5-diphosphate were added to aggregate platelets; the buffy coats were then gently rotated for 5 min and finally diluted in 150 ml balanced salt solution (BSS). The mononuclear cells were washed twice in BSS, resuspended in 175 ml RPMI 1640 (GIBCO, Paisley, UK) supplemented with 15–20% v/v human serum pool (not heat-inactivated) from eight healthy male donors, penicillin 100 U/ml, streptomycin 100 mg/ml, and L-glutamine 2 mmol/l. They were then incubated in five to six 260-ml culture flasks (Nunc, Roskilde, Denmark) at 37°C in 5% CO_2 and 95% atmospheric air. After 1·5 h of incubation the supernatants were decanted, and remaining non-adherent cells were removed using BSS (40 ml × 2) and gently shaken. Adherent cells (enriched in monocytes) were loosened mechanically after 1 h at 0°C, washed, and subsequently adjusted to 1 × 10^6 cells/ml in Krebs Ringer phosphate with 5 mM glucose, or resuspended in fresh RPMI 1640 medium as indicated above and incubated in microtitre plates. Cells were more than 95% viable as judged from trypan blue exclusion tests, and the preparation contained more than 90% monocytes (CD14^+). For cultivation, 8 × 10^5 cells were added to each well and incubated for 10 days at 37°C in 5% CO_2 and 95% atmospheric air. At day 9 some of the wells were supplemented with 100 U/ml IFN-γ (Schering-Plough Research, NJ), 100 pM GM-CSF (Schering Plough, Innishannon, Ireland), or 50 ng/ml IL-3 (Sandoz, Vienna, Austria). Cultivation of monocytes induces their differentiation into macrophages, as is shown by morphological, enzymatic, and receptor changes [13,14].

Assay for superoxide generation
Superoxide generation was assayed as superoxide dismutase (SOD) inhibitable reaction of cytochrome c (Sigma) in Krebs Ringer phosphate buffer supplemented with 5 mM glucose and 1 mg/ml cytochrome c [15]. Freshly isolated monocytes were assayed in solution, whereas cultivated monocytes were assayed as adherent cells, directly in the wells. The cells were stimulated by 1·6 μM phorbol-12-myristate-13-acetate (PMA) (Sigma) or by *P. carinii*, and incubated for 90 min in the presence or absence of 30 μg/ml SOD (Sigma, from bovine erythrocytes, S-2515). The basal level of superoxide generation was determined in the absence of stimulators. Reduction in cytochrome c was terminated by adding 10 μl of 3 μg/ml SOD to samples without this enzyme. Reduction in cytochrome c was measured spectrophotometrically as the change in absorbance at 550 nm. Reduction of cytochrome c by sodium dithionite (Merck, Darmstadt, Germany) was used to calculate superoxide production. As absorbance readings were linear in the area used, the amount of superoxide produced was

calculated as the fraction of change in absorbance in the experiments of the change produced by total reduction of the same amount of cytochrome c by sodium dithionite. This fraction was multiplied by the content of cytochrome c in the sample to express the number of moles of superoxide released.

Microscopy

Freshly isolated and cultivated monocytes incubated with *P. carinii* for various periods were fixed in 3% aqueous glutaraldehyde. After fixation for at least 2 h, post-fixation with 1% aqueous osmium tetroxide was performed. The cells were then dehydrated with ethanol, and finally embedded in Epon blocks placed on the culture slides. After polymerization, 1-μm sections were cut and stained with toluidine blue.

For the estimation of phagocytic activity, the percentage of cells with one or more phagocytic vacuole was calculated. An attempt to estimate the number of vacuoles in individual cells was not made.

For electron microscopy, selected survey sections were reembedded onto Epon blocks. These blocks were trimmed and 50 nm thick sections were cut. The sections were further counterstained with uranyl acetate and lead citrate. After mounting on copper grids the sections were examined and photographed by routine transmission electron microscopy.

Statistical analysis

Data were expressed as mean ± s.e.m., and differences between means were evaluated using Student's two tailed *t*-test. When multiple tests were performed the level of significance was changed according to the number of tests performed.

RESULTS

Superoxide production in monocytes

Freshly isolated monocytes produced superoxide ions when stimulated with opsonized pneumocysts. As shown in Fig. 1, superoxide generation was related to the number of *P. carinii* cysts added in the range 0.15×10^6–3.75×10^6. Above this level a relatively small increase was seen. When stimulated by 15×10^6 cysts, 1×10^6 monocytes produced 10.7 fmol O_2^-/cell per 90 min, compared with 3.0 fmol and 3.4 fmol released in response to tissue control and buffer, respectively. Superoxide production after stimulation with 3.75×10^6 opsonized pneumocysts (8.84 ± 1.25 fmol O_2^-/cell per 90 min) was significantly higher than the production induced by tissue control (3.0 ± 0.7 fmol O_2^-/cell per 90 min) ($P < 0.05$).

Superoxide production was dependent on opsonization, being in the same range as the response to tissue control and buffer, even when a large number of unopsonized cysts (15×10^6) were added (4.9 fmol O_2^-/cell per 90 min) (Fig. 2).

In order to normalize the results obtained with monocytes from different donors, O_2^- generation was expressed as percentage of the amount induced by PMA, a very potent activator of superoxide production [16]. Expressed in this way, pneumocysts opsonized in untreated serum stimulated monocytes to a production of superoxide that amounted to $26.6 \pm 1.58\%$ of the PMA response (10.7 fmole O_2^-/cell per 90 min) (Fig. 2). Opsonization in serum treated with 10 mM EDTA to bind Ca^{++} and Mg^{++} ions, thus blocking the complement activation, stimulated monocytes to a slightly larger extent, $31.5 \pm 2.31\%$ of the PMA response, whereas O_2^- production in
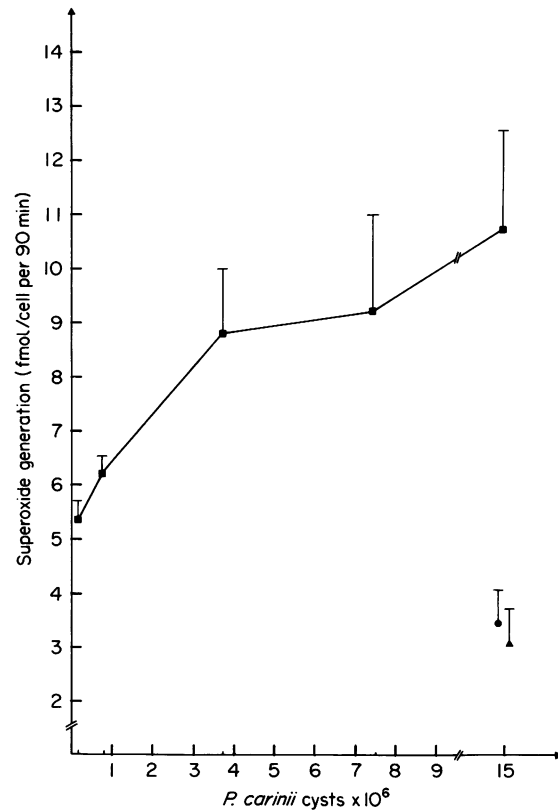

Fig. 1. Superoxide production by 1×10^6 monocytes incubated with the indicated number of *Pneumocystis carinii* cysts (■), Krebs Ringer phosphate buffer (●), or lung tissue (▲) adjusted to the same amount of protein as 15×10^6 *P. carinii* cysts. Each point represents the mean of three experiments (± s.e.m.).
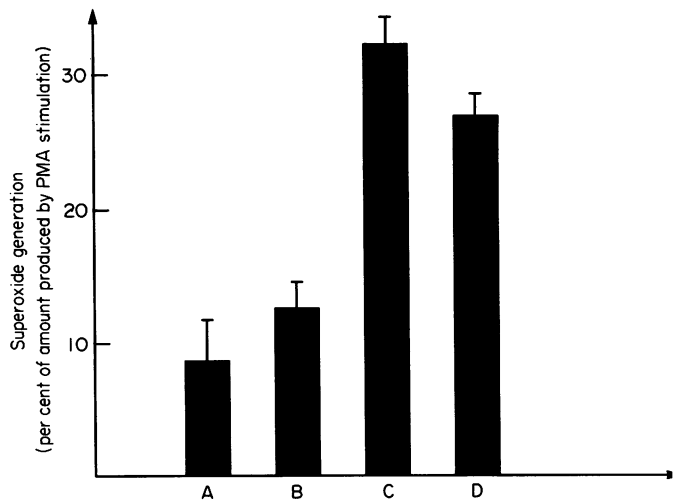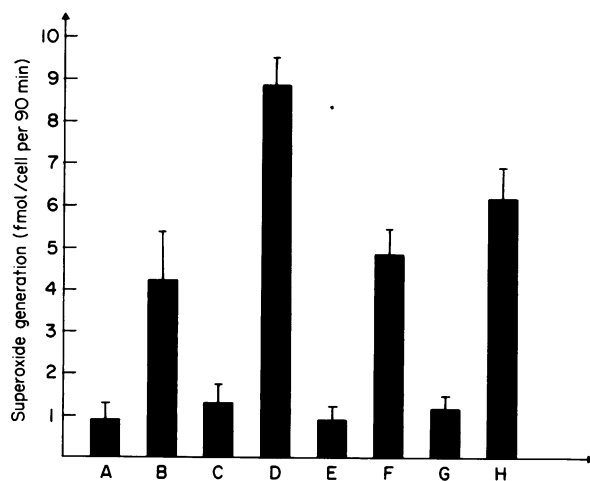

Fig. 2. Superoxide production by 1×10^6 monocytes incubated with Krebs Ringer phosphate buffer (A), 15×10^6 unopsonized *Pneumocystis carinii* cysts (B), 15×10^6 *P. carinii* cysts opsonized in EDTA-treated serum (C), and 15×10^6 *P. carinii* cysts opsonized in untreated serum (D). Each column represents the mean of three experiments (± s.e.m.). *$P < 0.01$. PMA, Phorbol myristate acetate.

monocytes stimulated with unopsonized pneumocysts was only $12 \pm 2.1\%$ of the PMA response. Compared with unopsonized pneumocysts, parasites opsonized in untreated serum induced a significantly higher O_2^- release ($P < 0.01$). The O_2^- response induced by *P. carinii* cysts opsonized in EDTA-treated serum was not significantly different from the O_2^- generation induced by cysts opsonized in untreated serum.

Superoxide production in monocyte-derived macrophages

Eight $\times 10^5$ monocytes were added to each well for cultivation, and the amount of O_2^- produced per plated cell, stimulated with 15×10^6 *P. carinii* cysts, was lower than the amount produced by freshly isolated monocytes (4.2 ± 1.2 versus 10.7 ± 1.8 fmol O_2^-/cell per 90 min) (Fig. 3). The O_2^- production in monocyte-derived macrophages incubated with pneumocysts was more than four-fold higher than the amount produced by cells incubated with Krebs Ringer phosphate buffer (4.2 ± 1.2 versus 0.9 ± 0.4 fmol O_2^-/cell per 90 min). Preincubation with 100 U/ml IFN-γ for 24 h significantly increased the superoxide generation induced by *P. carinii* to 8.8 ± 0.7 fmol O_2^-/cell per 90 min ($P < 0.025$). When PMA was used to stimulate the cells a large increase in O_2^- production was also seen in cells preincubated with IFN-γ (24.1 versus 14.2 fmol O_2^-/cell per 90 min in unprimed cells). Priming with 50 ng/ml IL-3 also enhanced O_2^- in cultivated monocytes stimulated with 15×10^6 *P. carinii* cysts, though less extensively (6.4 ± 0.7 versus 4.2 ± 1.2 fmol O_2^-/cell per 90 min) (NS). An effect on the PMA response was also seen (19.3 versus 14.2 fmol O_2^-/cell per 90 min). GM-CSF had only a small effect on superoxide production, amounting to 4.8 fmol O_2^-/cell per 90 min when pneumocysts were used to stimulate cells (NS) and to 15.0 fmol O_2^-/cell per 90 min when PMA was used.

Fig. 3. Superoxide production by monocyte-derived macrophages incubated with Krebs Ringer phosphate buffer or 15×10^6 *P. carinii*, without priming (A and B), primed with 100 U/ml IFN-γ for 24 h (C and D), primed with 100 pM granulocyte-macrophage colony-stimulating factor (GM-CSF) for 24 h (E and F), or with 50 ng/ml IL-3 for 24 h (G and H). The amount of superoxide expressed per plated cell. Each column represents the mean of six experiments ($\pm$s.e.m.). Superoxide production in IFN-γ-primed cells was significantly larger than in unprimed cells ($P < 0.025$). In GM-CSF and IL-3-primed cells no significant difference was found.

Microscopy

The ability of freshly isolated and cultivated monocytes to phagocytose pneumocysts was studied by light microscopy. Eight per cent of the monocytes incubated for 90 min with unopsonized pneumocysts had phagocytic vacuoles, whereas 16% and 13% of the monocytes had phagocytic vacuoles following incubation with 15×10^6 *P. carinii* cysts opsonized in EDTA-treated and -untreated serum, respectively.

Unprimed and IFN-γ-primed monocyte-derived macrophages appeared identical, with one or more prominent phagocytic vacuoles in about half of the cells after incubation with opsonized pneumocysts for 90 min (Fig. 4a). In one experiment, 43% of IFN-γ-primed cells had phagocytic vacuoles after 45 min, compared with 23% of unprimed cells (Table 1). After incubation for 90 min the difference between the two groups of cells was negligible (63% versus 70%), and additional incubation for up to 12 h gave only a small increase in the percentage of cells with phagocytic vacuoles, and there was no difference between unprimed and IFN-γ-primed cells.

Electron microscopic examination showed a somewhat heterogeneous population of monocyte-derived macrophages, with a well developed endoplasmatic reticulum and vacuoles in a large proportion of the cells. Unprimed and IFN-γ-primed cells appeared alike. Most vacuoles contained partly degraded pneumocysts (Fig. 4c) or debris of varying electron density, indicating a rapid and complete intracellular break-down of pneumocysts. Only a small fraction of the vacuoles contained intact or slightly degraded cysts of *P. carinii* (Fig. 4c,d).

DISCUSSION

We have shown that monocytes are able to phagocytose cysts of *P. carinii* and activate the respiratory burst during this process, provided that the microorganisms have been opsonized. Rat lung tissue prepared and opsonized in the same way as pneumocysts and adjusted to the same protein content as 15×10^6 pneumocysts could not stimulate the cells, suggesting that the effect was specific and not caused by lung tissue inevitably trapped in the pneumocyst suspension. Pneumocysts used in this study came from a rat source, and although species differences between pneumocysts from different hosts can be demonstrated using MoAbs [17], cross-reactivity between rat and human pneumocysts has been found [18,19]. Only pneumocysts opsonized with serum could activate the respiratory burst in monocytes. A similar dependence on opsonization of pneumocysts with serum has been found in granulocytes [5,20] but a higher O_2^- production was reported in this cell type [20]. Antibodies were probably reponsible for this effect, since blocking the complement cascade with EDTA did not decrease the effect on O_2^- production. However, the effect of other opsonins, insensitive to the removal of magnesium and calcium ions, could also be responsible.

In previous studies we found that AIDS patients with PCP had a lower level of specific IgG and a higher level of specific IgA antibodies against *P. carinii*, both in serum and bronchial lavage fluid, than controls [21,22]. These and the present results suggest that phagocytic cells in conjunction with IgG antibodies might be important in the defence against pneumocysts.

To study the interaction between pneumocysts and macrophages, monocyte-derived macrophages were used. These cells preserved the ability to phagocytose pneumocysts and produce
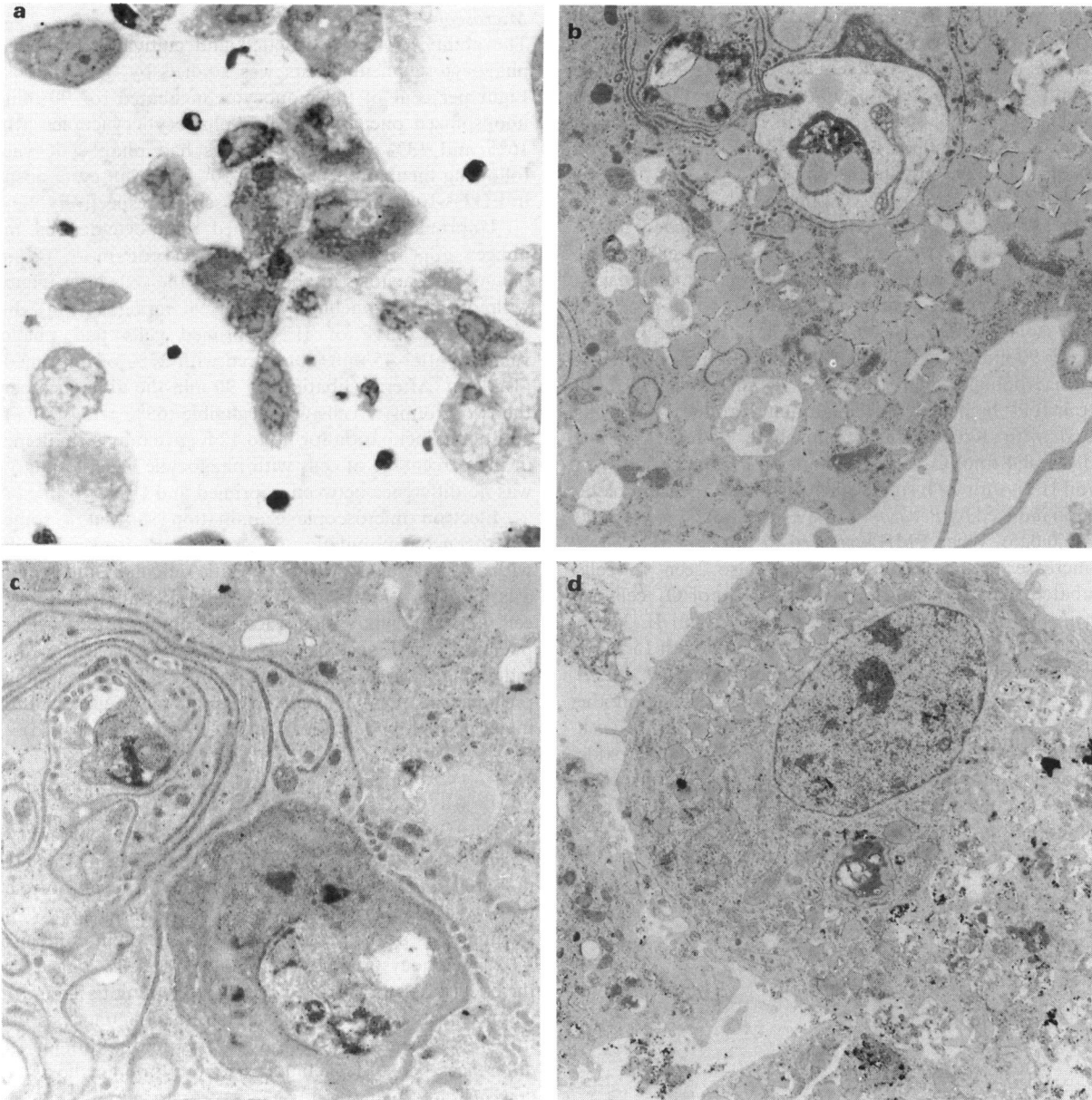
Fig. 4. (a) The light microscopic appearance of unprimed monocyte-derived macrophages incubated with opsonized pneumocysts for 90 min. Large phagocytic vacuoles with stained material are seen in half of the cells. (b-d) The electron microscopic appearance of monocyte-derived macrophages unprimed (c), and primed with 100 U/ml IFN-γ for 24 h (b,d). In b, a large vacuole containing relatively intact *Pneumocystis carinii* cyst material is seen. A more advanced stage of degradation is shown in c, in which the semilunar shape of a *P. carinii* cyst can still be identified. The general appearance of cultivated monocytes characterized by abundant rough endoplasmatic reticulum is shown in d. A phagocytic vacuole containing a *P. carinii* cyst can be identified below the nucleus. Original magnifications: (a) ×200; (b) ×10000; (c) ×12000; (d) ×2500.

superoxide ions, though the latter were reduced compared with the use of freshly isolated monocytes. The reduction in respiratory burst activity during differentiation to macrophages has been described by others [23].

Priming of macrophages with both IFN-γ and IL-3 for 24 h increased O_2^- production induced by pneumocysts. The effect of IFN-γ seemed to be mediated both through an increased phagocytosis, since a two-fold higher percentage of cells with phagocytic vacuoles was seen in IFN-γ-treated cells after 45

min, and through an increased potential of superoxide production, since the PMA response was also increased in IFN-γ-treated cells. The ability of macrophages to activate the respiratory burst when challenged with pneumocysts is in agreement with the findings of Hidalgo *et al.* [6] and Taylor *et al.* [5], who used a macrophage cell line and alveolar macrophages from rats and rat serum for opsonization.

The effect of the three cytokines used here has been studied in macrophages for the effect on biostatic activity, using

	Time of incubation			
	45 min	90 min	4 h	12 h
Unprimed cells	23	63	57	69
IFN-γ-primed cells	43	70	70	77

The percentage of 8×10^5 plated monocyte-derived macrophages with one or more phagocytic vacuoles after incubation with 15×10^6 *Pneumocystis carinii* for the indicated periods. Cells were unprimed or primed for 24 h with 100 U/ml IFN-γ.

microorganisms other than pneumocysts to stimulate the cells. Thus, fungicidal activity has been found to be dependent on IFN-γ [24], whereas this cytokine alone was not sufficient to induce bactericidal activity against *Mycobacterium avium* [25]. In support of the importance of IFN-γ in the defence against *P. carinii*, Beck et al. [26] found that this cytokine, administered as an aerosol, significantly reduced the intensity of infection with *P. carinii* in mice.

PCP is an important infection in HIV-infected patients, and although the serum level of IFN-γ in HIV-infected patients seems to be increased [27,28], most reports have shown a reduced ability of lymphocytes from HIV-infected patients to produce IFN-γ on stimulation with mitogen [7] and microorganisms [8]. Such a decreased ability to produce IFN-γ on demand might contribute to the liability of HIV-infected patients to have PCP.

As for IL-3, an effect on activation of the respiratory burst in macrophages and a biostatic effect against leishmania, trypanosomes, and *Histoplasma capsulatum*, have been demonstrated previously [10, 29]. We found a less convincing effect of IL-3. One explanation could be longer cultivation of cells (10 *versus* 3 days) and shorter preincubation time (1 *versus* 3 days) in our study. GM-CSF increased fungicidal activity [29], but the effect depended on the cytokine being present throughout the period of cultivation; preincubation for 24 h had no effect. On the other hand this length of time was sufficient to prime monocyte-derived macrophages to give increased O_2^- production when mycobacteria were used as the stimulant [7].

The phagocytic and degrading ability of monocyte-derived macrophages, as studied by light and electron microscopy, indicates that these cells are indeed able to engulf and degrade pneumocysts. The latter process seems to be fast and efficient, since most microorganisms were partly or fully degraded after 90 min. These results parallel the findings of Wehle et al. [30], who studied bronchoalveolar lavage fluids from AIDS and non-AIDS patients with PCP. They found that degraded products of *P. carinii* by far outnumbered intact cysts in alveolar macrophages. Interestingly, in that study, AIDS patients had a significantly higher proportion of intact cysts, indicating a defective ability to degrade the pneumocysts.

In conclusion, our results show that opsonized monocytes and macrophages have the ability to phagocytose pneumocysts, to respond with activation of the respiratory burst, and to degrade the microorganisms. In macrophages, activation of the respiratory burst was significantly increased when the cells had

been primed with IFN-γ, and to a lesser extent when primed with IL-3. GM-CSF had only a small effect on superoxide production.

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