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Killing of pathogens associated with chronic granulomatous disease by the non-oxidative microbicidal mechanisms of human neutrophils

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Summary. The susceptibility of opportunist pathogens associated with chronic granulomatous disease (CGD) to the non-oxidative killing mechanisms of neutrophils has been assessed by incubation in human neutrophil primary granule lysate. The dose and pH-dependency of killing of *Aspergillus fumigatus, Candida albicans, Escherichia coli, Nocardia asteroides, Serratia marcescens* and *Staphylococcus aureus* differed markedly and may partly explain their virulence in CGD, in which oxygen-dependent killing mechanisms are defective. At the acid pH in CGD neutrophil phagosomes *S. aureus, Ser. marcescens, N. asteroides* and *A. fumigatus* spores were highly resistant but *C. albicans*, a less frequent pathogen in patients with CGD, was much more susceptible.

Introduction

Neutrophil polymorphonuclear leucocytes kill micro-organisms by oxygen-dependent and oxygen-independent mechanisms. In the former, oxygen is consumed in the respiratory burst to produce hydrogen peroxide and other microbicidal compounds such as hypochlorous acid and chloramines, whereas oxygen-independent killing depends on the delivery into the phagosome of pre-formed antimicrobial compounds stored in the neutrophil granules. The mechanisms of both oxygen-dependent[1] and oxygen-independent killing[2] have recently been reviewed.

The respiratory burst is required for optimal antimicrobial function by neutrophils. Its importance is demonstrated by chronic granulomatous disease (CGD), a group of rare conditions[3] in which predisposition to severe systemic infection results from absence of the respiratory burst.[4,5] Neutrophils from patients with CGD have impaired microbicidal activity and digestion *in vitro* but are able to kill significant numbers of bacteria.[5–7] Similarly, normal neutrophils under anaerobic conditions *in vitro* kill some bacterial species efficiently in spite of impaired antimicrobial activity against others.[8,9] This microbial killing by oxygen-independent mechanisms is thought to be of importance in inflammatory environments where oxygen tension is low.

Neutrophil microbicidal compounds that act independently of the respiratory burst include bactericidal permeability-increasing factor (BPI),[10] cathepsin G (formerly known as chymotrypsin-like cationic protein),[11] the defensins,[12,13] lactoferrin,[14] and lysozyme.[15] In addition to these specific compounds there are several other less well characterised compounds such as cationic proteins of mol. wt 57 000,[16] 55 000[17] and 37 000[16] and some degradative enzymes with microbicidal activity.[18,19] The effectiveness of these compounds *in vivo* depends on alkalinisation of the phagosome by the neutrophil respiratory burst.[7,20,21] CGD neutrophils, without a respiratory burst, fail to kill some microbial species because they rely on non-oxidative killing which is rendered less effective by poor phagosomal pH control.[7] As a result, patients with CGD have increased susceptibility to infection, in particular with *Staphylococcus aureus, Serratia marcescens, Aspergillus* spp., *Salmonella typhimurium* and, more rarely, *Escherichia coli* and *Candida* spp.[22–24]

We have attempted to explain the increased virulence of some of these pathogens in CGD by assessing their susceptibility to neutrophil granule lysate *in vitro*. This model of non-oxidative killing imitates the CGD neutrophil phagosome into which the granule contents are liberated by degranulation[2] at low pH,[7] independently of a respiratory burst.[4,5]

Materials and methods

Micro-organisms

All micro-organisms except *A. fumigatus* were human clinical isolates kindly provided by the Departments of Medical Microbiology, University College

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Hospital and Guy's Hospital, London, where they were typed by routine microbiological techniques.

Isolates of the following micro-organisms were used: *S. aureus* (Oxford strain and one wound isolate), *Ser. marcescens* (two wound isolates), *E. coli* (two gastric isolates of smooth colonial morphology), *Nocardia asteroides* (two pulmonary isolates) and *C. albicans* (one oral and one vaginal isolate). The micro-organisms were maintained on blood-agar plates and grown to mid-exponential phase by dilution of an overnight culture 1 in 100 in fresh Nutrient Broth (Oxoid). After culture at 37°C with vigorous shaking, the micro-organisms were harvested and washed twice by centrifugation (13 000 *g* for 1 min).

Two pulmonary isolates of *A. fumigatus* (kindly provided by Dr C. Campbell, Mycological Reference Laboratory, Central Public Health Laboratory, 61 Colindale Avenue, London) were maintained on Malt Extract Agar (Oxoid). Cultures 4–5 days old were washed with Tween 20 0·05% in water, and spores were harvested after sedimentation of hyphae for 30 min and filtration through filter paper (Whatman no. 1). Spores were stored at −20°C.

Suspensions of micro-organisms and spores in water were adjusted to known density by spectrophotometry at 600 nm.

Preparation of human neutrophils

Normal human neutrophils were separated from buffy coat residues (National Blood Transfusion Service) by dextran sedimentation and Ficoll-Hypaque centrifugation after dilution with an equal volume of isotonic saline. Residual erythrocytes were removed by hypotonic lysis in water for 15 s to give a suspension containing more than 95% neutrophils. All further procedures were performed at 4°C.

Preparation of primary granules and granule extract

Neutrophils were suspended in sucrose 6% w/v and disrupted by nitrogen cavitation at 7×10^6 Pa after equilibration for 1 h. A post-nuclear supernate (230 *g* for 30 min) was fractionated by sucrose density gradient centrifugation as previously described.[25] Primary granules banded at a specific gravity of 1·225 g/ml and were identified by the marker enzyme myeloperoxidase (MPO: E.C. 1.11.1.7) as measured by guaiacol peroxidation.[26]

Primary granules were washed three times by centrifugation (100 000 *g* for 30 min) and extracted in 0·2 M sodium acetate–acetic acid, pH 4·0, at 4°C for 20 h in a rotating tube.[27] Insoluble material was removed by centrifugation (1430 *g* for 10 min followed by 100 000 *g* for 30 min) and the supernate was dialysed extensively against 154 mM NaCl with benzoylated dialysis membrane (mol. wt cut-off 1000; Sigma) to retain low mol. wt components. Granule extract was stored at −20°C at a final concentration of 1·8 mg/ml. Granule extract was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).[28]

To determine the identity of components of the granule extract responsible for the killing of *S. aureus* observed at alkaline pH, the extract was fractionated into four peaks by gel filtration on Sephacryl S100.[29] Granule extract (0·5 ml) was separated on a column 300 × 20 mm equilibrated with 145 mM NaCl buffered with 10 mM phosphate buffer, pH 7·5. Fractions from each peak were tested for activity at pH 7·5. Protein assays were performed with the Pierce BCA system (Pierce, Rockford, IL, USA).

Killing assays

Killing assays were performed as previously described.[30] Micro-organisms were suspended at a final concentration of 10^6 cfu/ml in 10 mM citrate phosphate buffer, pH 7,[31] with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 113 mM NaCl supplemented with neutrophil primary granule extract at various concentrations. Fractions of granule extract were tested against micro-organisms adjusted to a final concentration of 10^5 cfu/ml with bovine serum albumin 0·2 mg/ml. The reaction volume was usually 500 µl, but some assays were performed in 50 µl. To investigate the pH dependency of killing, the buffer was adjusted to pH values from 5·5 to 8·0. Suspensions were incubated, with mixing, at 37°C for 60 min and surviving micro-organisms were counted by serial dilution and colony counting on suitable media by routine methods.[32] Results were calculated as the mean and SEM from at least three experiments with colony counts performed in triplicate or quadruplicate for each sample and expressed as a percentage of the original viability, as determined by control assays performed in buffer alone. The pH remained stable during assays to within 0·15 pH units of the starting pH. Statistical comparisons were made with Student's *t* test.

Results

Extracted proteins were representative of whole granules, as judged by SDS-PAGE (data not shown), reflecting the high efficiency of this extraction procedure.[33] Granule extract showed antimicrobial activity against some species with the potency highly dependent on the assay conditions. The dose dependency of killing at pH 7·0 is shown in fig. 1. Different micro-organisms showed different susceptibility to granule extracts. *S. aureus* and *E. coli* showed a threshold effect, a significant reduction of viability occurring over a small dose range. The killing of *C. albicans* was directly proportional to dose over a much wider dose range. *A. fumigatus* spores, *Ser. marcescens* and *N. asteroides* were highly resistant to granule extract.

The pH dependency of killing for each strain is shown in fig. 2. *E. coli* and *C. albicans* were more

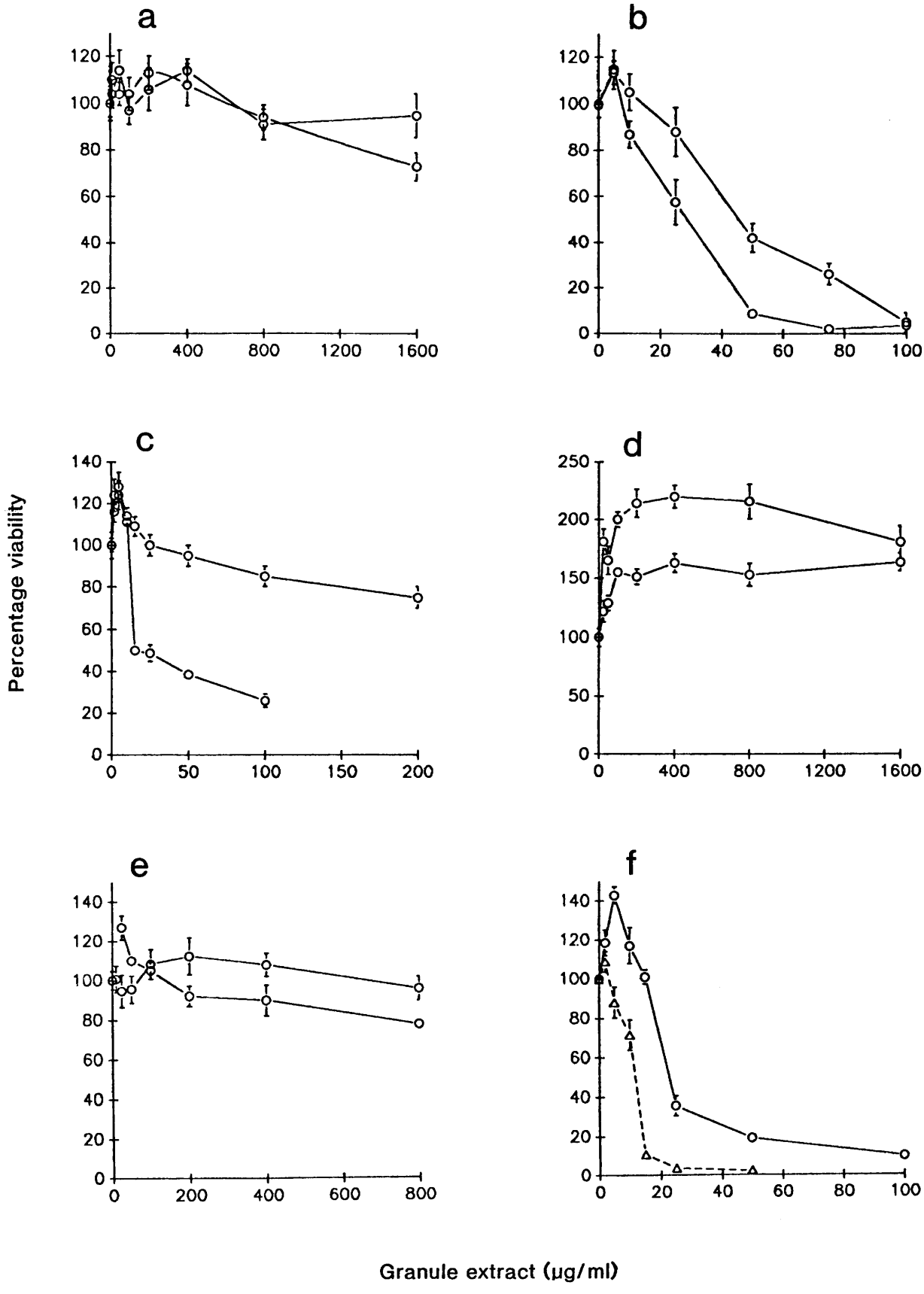

Fig. 1. Each graph shows the killing of two strains of each micro-organism (10^6 cfu/ml) exposed to various doses of granule extract at 37°C for 60 min at pH 7·0. The mean and SEM of three experiments is shown. Colony counts were performed in quadruplicate. Changes in viability greater than 50% were always significant ($p \leq 0{\cdot}05$). (**a**) *A. fumigatus*; (**b**) *C. albicans*; (**c**) *E. coli*; (**d**) *N. asteroides*; (**e**) *Ser. marcescens*; (**f**) *S. aureus* (- - -, Oxford strain).

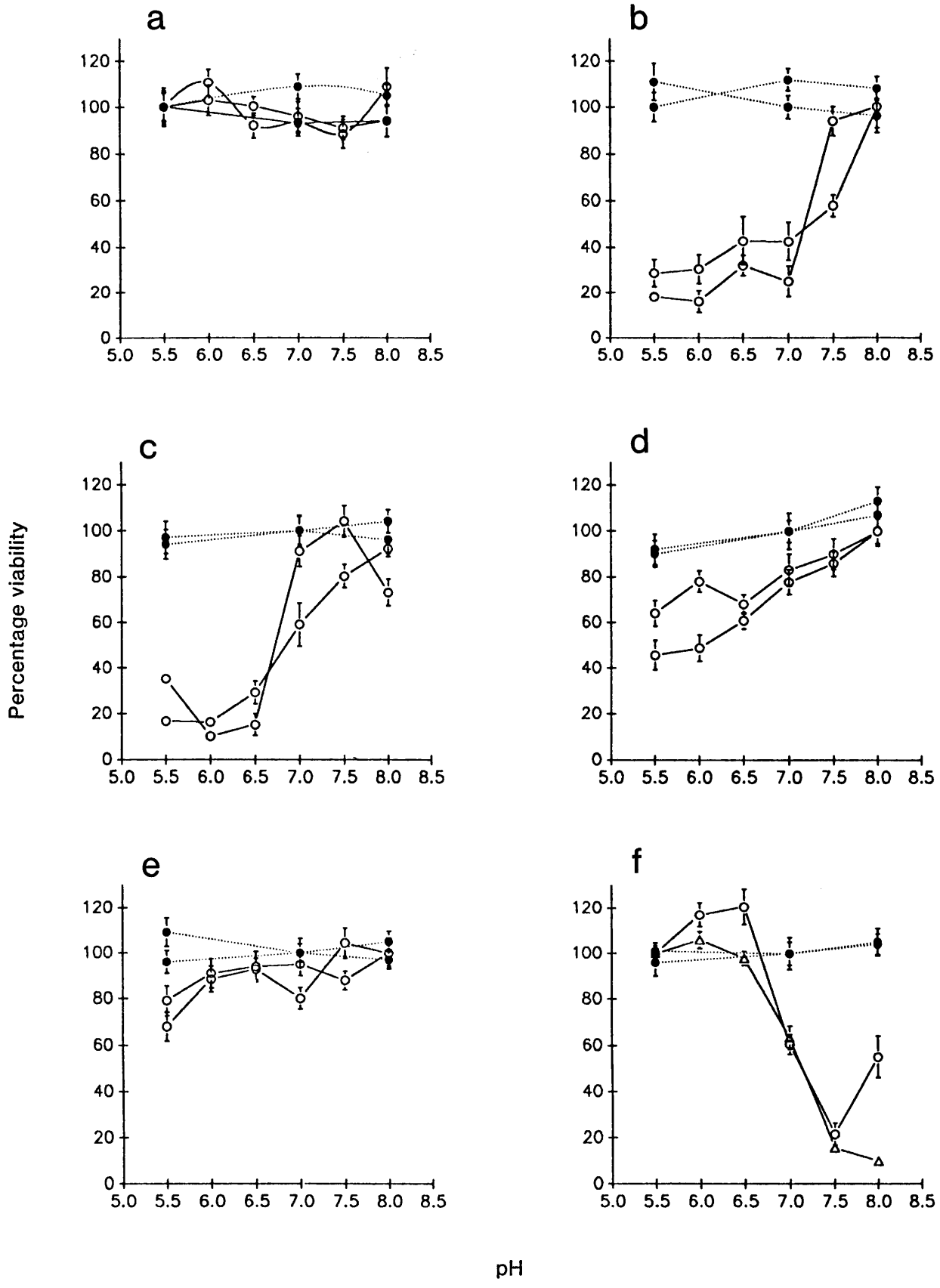

Fig. 2. Each graph shows the effect of pH on the killing of two strains of each micro-organism (10^6 cfu/ml) by the following fixed doses of granule extract at 37°C for 60 min (○—○): **(a)** *A. fumigatus* (800 μg/ml), **(b)** *C. albicans* (50 μg/ml), **(c)** *E. coli* (20 μg/ml), **(d)** *N. asteroides* (400 μg/ml), **(e)** *Ser. marcescens* (400 μg/ml), **(f)** *S. aureus* (fresh isolate 25 μg/ml; Oxford strain 15 μg/ml); ●····●, the effect of buffer alone. The mean and SEM of three experiments is shown. Colony counts were performed in quadruplicate. Changes in viability greater than 50% are significant ($p \leq 0{\cdot}05$).

susceptible to granule extract at acid pH, whereas strains of *S. aureus* were resistant to extract at acid pH and susceptible only at neutral and alkaline pH. *Ser. marcescens* was very slightly more susceptible at acid pH. *N. asteroides* was susceptible only at acid pH, and then only at very high concentrations of extract. *A. fumigatus* spores were resistant at each pH value tested.

The bactericidal effects of extract fractions against *S. aureus* at alkaline pH are shown in fig. 3.

Discussion

In normal neutrophils, micro-organisms are killed by the combined action of oxygen-dependent and oxygen-independent mechanisms. CGD neutrophils possess only oxygen-independent mechanisms and killing depends on the microbicidal effect of the neutrophil granule proteins. An attempt was made to correlate resistance to neutrophil granule proteins with the virulence of some of the opportunist pathogens associated with CGD.

It is not easy to associate specific pathogens with CGD by comparing frequency of infection with the normal population, because the number of cases is small and the clinical course variable. *S. aureus* and *Ser. marcescens* are isolated frequently and are particularly troublesome.[22,23] Other pathogens reported include *Klebsiella* spp., *Pseudomonas aeruginosa, S. albus, Aspergillus* spp., *N. asteroides, E. coli* and *Salmonella* spp. but increased virulence cannot be inferred without further evidence of the unusual sites and severity of infection seen in CGD. The susceptibility of six micro-organisms whose pathogenicity is enhanced in CGD was tested—*A. fumigatus* spores, clinical isolates of *N. asteroides, S. aureus*, and *Ser. marcescens*, and the *S. aureus* Oxford strain which CGD neutrophils kill *in vitro* with reduced efficiency. *C. albicans* blastospores and *E. coli* were also tested because, although they are not normally associated with CGD, oxygen-dependent mechanisms may be required for normal neutrophils to kill then.

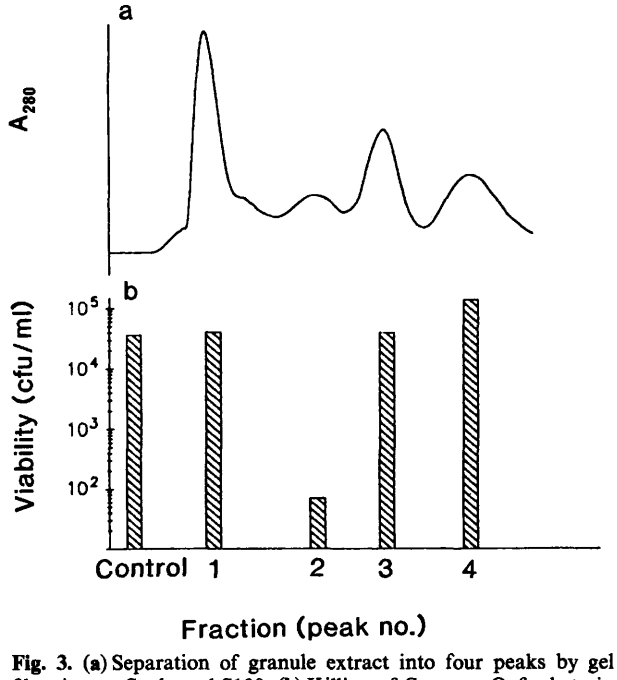

Fig. 3. (**a**) Separation of granule extract into four peaks by gel filtration on Sephacryl S100. (**b**) Killing of *S. aureus* Oxford strain by fractions from each peak. The means of four experiments are shown. Colony counts were performed in quadruplicate. Final concentrations of protein incubated with 10^5 cfu/ml were: peak 1, 173 μg/ml; peak 2, 85 μg/ml; peak 3, 103 μg/ml; peak 4, 90 μg/ml.

Extraction of neutrophil primary granules in acetate solubilises granule components efficiently[33] and the extract used was potently antimicrobial when tested against some of these micro-organisms, the pattern reflecting their virulence in CGD. Each organism shows an apparent increase in viability at some doses. This is a non-specific protein effect caused by the highly charged granule proteins binding to the micro-organism surface which alters the degree of bacterial clumping and adsorption to the assay tube wall.

Optimal killing of *S. aureus* by non-oxidative mechanisms is known to require the respiratory burst.[7,34] Both strains of *S. aureus* tested were susceptible to granule extract only at neutral or alkaline pH. This neutral pH optimum for killing has been noted by others[30] and may explain the pathogenicity of *S. aureus*, both in CGD[7] and at foci of anaerobic infection.[35] *S. aureus* is killed at acid pH only in the presence of a respiratory burst because the MPO system has an acid pH optimum.[36] Fractionation of the granule extract by gel filtration showed that almost all the activity against *S. aureus* at pH 7·5 was retrieved in peak 2. The major components of peak 2 are the neutral proteases elastase and cathepsin G[37] but small amounts of BPI, the 37 000-mol. wt cationic protein and other unknown factors are probably also present. Further studies will be necessary to determine the individual components responsible for the killing.

Ser. marcescens, N. asteroides and *A. fumigatus* were markedly resistant to granule extract. Protection against *Ser. marcescens* is mediated in part by neutrophils[38] and *Ser. marcescens* is a frequent pathogen in CGD. Its killing of *Ser. marcescens* is delayed in MPO-deficient neutrophils[39] and impaired in CGD neutrophils,[34] which points to the importance of the MPO system. The strains used in the current investigation were highly resistant to granule extract at every pH value tested and this may explain the pathogenicity of this species in CGD, where MPO and oxygen-dependent killing systems are defective.

N. asteroides is also associated with infection in CGD and is highly resistant to killing by neutrophils and monocytes,[40] although neutrophils can exert a bacteriostatic effect by preventing filament formation.[41] This effect is oxygen-independent and is normally exerted by CGD neutrophils and by granule extract *in vitro*.[41] Resistance to killing may also be due partly to inhibition of degranulation[42] and of acidification.[43] In these studies, *N. asteroides* was found to be extremely resistant to killing by granule extract but the techniques used in the present study do not detect

the bacteriostatic effects previously reported.[41] Thus, although other mechanisms may confer pathogenicity on *N. asteroides* it is clearly resistant to neutrophil non-oxidative killing.

A. fumigatus and *C. albicans* are opportunist pathogens and, therefore, likely to be susceptible to killing by neutrophils.[44] The susceptibility of *Aspergillus* spp. to killing by neutrophils depends on the stage of the life cycle. Conidia stimulate neutrophils poorly and are highly resistant to oxidative[45] and non-oxidative[46] killing mechanisms. They are more easily killed by resident macrophages from some sites,[47] or during growth phases.[45,46] The hyphal form is more easily killed by neutrophils,[48] and rabbit[46] and human[49] neutrophil granule lysates are also effective, though slightly less efficient. Neutrophils from patients with CGD or MPO deficiency are less able to damage hyphal forms than normal neutrophils.[49] In the present investigation, *A. fumigatus* spores were shown to be highly resistant to granule extract, thus confirming previous results.[46] However, it is unclear whether the resistance of the spores might be important in initiating infection in patients with CGD. Survival of *A. fumigatus* spores *in vitro* may be further aided by uncharacterised diffusible factors which have been reported to inhibit neutrophil function.[50]

The killing of *Candida* spp. by human neutrophils has been reported to depend on both MPO and the respiratory burst, as judged by CGD neutrophils, anaerobic culture[51] and MPO deficiency.[39,52] However, *Candida* spp. are susceptible to non-oxidative killing by cathepsin G, defensins and lysozyme. The killing of *C. albicans* reported here was directly dose-related over a wide range of doses, as previously reported.[53] *C. albicans* was killed best at low pH, which suggests that defensins alone do not mediate the effect.[54] This pH dependency may explain why these micro-organisms are not common pathogens in CGD, because they would be killed by granule components in the more acid phagosome.

E. coli is reported to cause frequent systemic infections in CGD.[22,23] There are marked differences in the susceptibility to non-oxidative killing within the species, reflecting the potency and target specificity of BPI.[10] *E. coli* can be susceptible to BPI, defensins, and the 57 000- and 37 000-mol. wt cationic antimicrobial proteins and, to a lesser degree, lysozyme, although smooth strains are generally resistant to BPI.[55] In view of the known potency of BPI towards *E. coli*, it would be expected that smooth strains would be more pathogenic in CGD, but such a distinction between strains is not made in reported studies. The strains of *E. coli* used in these experiments showed marked variation in susceptibility to granule extract, although the effect of pH was similar for both. Since killing by crude lysate is optimal at acid pH rather than at neutrality, it is unlikely that BPI is responsible for the observed killing.

The pathogenicity of micro-organisms is multifactorial and depends on their ability to evade all the host defence systems *in vivo*, not just the neutrophil non-oxidative mechanisms. However, the results of these investigations suggest that the susceptibility and pH dependence of granule-extract mediated killing may help explain microbial virulence in CGD where killing depends on non-oxidative mechanisms alone. *S. aureus* clearly requires neutral pH for effective non-oxidative killing and is resistant at the acid pH found in the phagosomes of CGD neutrophils. This finding lends support to the suggestion that an important function of the respiratory burst is to control phagosomal pH. Species such as *Ser. marcescens, N. asteroides* and *A. fumigatus* are highly resistant to granule extract over a wide pH range and would be resistant to killing in CGD neutrophils. These species are presumably killed efficiently by oxidative mechanisms in normal neutrophils as they are uncommon pathogens in the normal population. *C. albicans* may be an uncommon pathogen in CGD because it is susceptible to non-oxidative killing at the acid pH found in the CGD neutrophil phagosome.

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