THE ACTION OF CELLS FROM PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE ON STAPHYLOCOCCUS AUREUS

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SUMMARY. Contrary to current opinion, neutrophil leucocytes from patients with the inherited immunodeficiency syndrome chronic granulomatous disease (CGD) killed 80% of ingested Staphylococcus aureus. Bacterial killing was not impaired by increasing the ratio of bacteria to cells from 1:1 to 10:1. The organisms that survived within the patients' cells did not themselves appear to constitute an unduly resistant subpopulation because they were killed when exposed to fresh cells, and no growth phase of a synchronous culture was found to be particularly resistant. The pH within the phagocytic vacuoles of CGD neutrophils and monocytes is abnormally low and methylamine, which has been shown to normalise this vacuolar pH, improved killing. Clumped bacteria appeared to be more resistant to killing than dispersed ones, suggesting that organisms near the centre of a clump might be protected from the toxicity of the compromised killing systems in cells of these patients.

INTRODUCTION

Phagocytosis of bacteria by leucocytes is accompanied by a burst of non-mitochondrial respiration (Baldridge and Gerard, 1933). Although this oxygen consumption is not required for phagocytosis (Selvaraj and Sbarra, 1966) it is required for the killing of certain bacteria (Selvaraj and Sbarra, 1966; McRipley and Sbarra, 1967; Mandell, 1974). There are two major pieces of evidence linking the respiratory burst to bacterial killing. Patients with the rare congenital syndrome of chronic granulomatous disease (CGD), whose leucocytes do not show any evidence of the respiratory burst (Holmes, Page and Good, 1967), develop unusually frequent and severe infections, often with unusual organisms, and their cells kill bacteria less efficiently than normal (Quie et al., 1967; Mandell and Hook, 1969a). In addition, the killing of certain bacteria by normal leucocytes is impaired in anaerobic conditions.

The respiratory burst has been thought to promote bacterial killing by the generation of free toxic radicals and hydrogen peroxide which could function as the substrate for myeloperoxidase-mediated chlorination (reviewed by Klebanoff, 1975;
Babior, 1978). It is generally accepted that bacteria that contain catalase are highly resistant to killing by cells from patients with CGD because they are able to destroy traces of \( \text{H}_2\text{O}_2 \) that might accumulate, whereas organisms that produce \( \text{H}_2\text{O}_2 \) generate the instrument of their own destruction (Mandell and Hook, 1969b; Mandell, 1975). In this study we have examined the efficiency of intracellular killing of \textit{Staphylococcus aureus} by neutrophils from patients with CGD.

**Materials and methods**

The patients were five severely affected males with X-linked CGD, all of whose cells lacked spectral evidence of cytochrome b \(-245\) and a mildly affected girl with the autosomal recessive variant (Segal and Jones, 1980).

**Isolation of cells.** Neutrophils were isolated from peripheral blood by dextran sedimentation of erythrocytes, centrifugation through ficoll/sodium metrizoate and haemolytic lysis of residual erythrocytes and then suspended in RPMI 1640 Medium (Flow Laboratories, PO Box 17, Second Avenue, Irvine, Ayrshire KA12 8NB) containing preservative-free heparin 5 IU/ml (Weddel Pharmaceuticals, Weddel House, 14 West Smithfield, London EC1A 9HY). Cell preparations from control subjects were 95–100% pure neutrophils whereas those from the patients contained 5–10% eosinophils.

**Radiolabelling and opsonisation of bacteria.** A reference culture of \textit{S. aureus} (Oxford strain, NCTC6571) was maintained on an agar slope. An overnight culture of the organisms in broth (12 ml of Oxoid Nutrient Broth No. 2) containing \( \text{H}^3 \)-glucose 25 \( \mu \text{Ci} \) (The Radiochemical Centre, White Lion Road, Amersham, Bucks HP7 9LL) was centrifuged at 5000 g for 20 min at 4°C and resuspended in 1 ml of RPMI 1640 medium containing heparin 5 IU/ml, pooled, partially purified human immunoglobulins \( 200 \text{ mg/ml} \) (Lister Institute, Elstree, Herts) and 50 mm tricine buffer \( \text{pH} \) 8.5. After 15 min at \( 37°C \) the bacteria were centrifuged at 7000 g for 4 min, washed and then carefully resuspended in RPMI 1640 medium containing heparin 5 IU/ml by repeated aspiration into and expression through a 23-gauge needle until all bacterial clumping had been eliminated. Bacteria were diluted and counted microscopically in a Thoma counting chamber (Weber Scientific International Ltd, Lancing, Sussex).

**Growth of bacteria in synchronous culture** was by the methods of Mitchison and Vincent (1965) and Chatterjee, Taber and Young (1971). A medium containing neutralised soya peptone (Oxoid L44) 5 g/L, yeast extract (Oxoid L21) 5 g/L, \( \text{K}_2\text{HPO}_4 \) 3 g/L, glucose 2 g/L and \( \text{NaCl} \) 2 g/L, \( \text{pH} \) 7.2 was autoclaved for 15 min. Conditioned growth medium (CGM) was prepared by incubating 250 ml of this medium with 2 ml of an overnight bacterial culture for 3 h without shaking at \( 37°C \) in an Erlenmeyer flask. This suspension was then centrifuged at 7000 g for 10 min at 20°C and the supernate was stored at \(-20°C\). A 100-ml sample of CGM was warmed to \( 37°C \), inoculated with 2 ml of an overnight culture and incubated at \( 37°C \) for 4 h in a shaking water bath. After centrifugation at 600 g for 5 min at \( 25°C \), 2 ml of the pellet was suspended in 2 ml of distilled water, layered on the surface of a 19-ml linear sucrose-density gradient containing 2–12\% (w/w) sucrose in water, and centrifuged at 4°C for 5 min (\( g \) minimum = 110, maximum = 300). Most of the bacteria were either pelleted or formed a band about half way down the tube. The uppermost 3 ml of the gradient, which was slightly turbid, was aspirated and inoculated into CGM containing \( \text{H}^3 \)-glucose 25 \( \mu \text{Ci/ml} \) in a shaking water bath at \( 37°C \). Samples were taken every 10 min from 15 to 75 min. Numbers were determined by plate counts and bacteria were opsonised as described above, except that they were opsonised on ice, rather than at \( 37°C \), and for 60 min.

**Incubation of bacteria with cells.** Of a suspension of neutrophils (\( 1 \times 10^8 \) cells/ml of RPMI 1640 medium) containing heparin 5 IU/ml, 0-1 ml was incubated in the rapidly stirred, thermostatically controlled plastic chamber of an oxygen electrode (Rank Bros., Bottisham, Cambs). A 0-1-ml sample of opsonised bacteria was then added to the cells in a ratio of 1:1 (in some experiments the ratio was 10 bacteria/cell). At timed intervals from 15 sec to 16 min, 50-\( \mu \text{l} \) samples were taken for assessment of bacterial phagocytosis and viability.

**Assessment of bacterial phagocytosis.** A 50-\( \mu \text{l} \) sample of the incubation mixture was taken into 1 ml of RPMI 1640 medium at 4°C containing lysostaphin (Sigma) 100 \( \mu \text{g/ml} \) to lyse...
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extracellular bacteria (Tan, Watanakunakorn and Phair, 1971) and 1 mM n-ethyl maleimide to prevent further phagocytosis during the subsequent incubation. After 10 min the cells were suspended by mixing on a vortex mixer, incubated at 37°C for 15 min and centrifuged at 7000 g for 4 min. (Microfuge B, Beckman Instruments, Turnpike Road, HighWycombe, Bucks.) A 500-μl sample of the supernate was taken into 5 ml of a triton-toluene based scintillation fluid and counted in a β-scintillation counter. A sample of the original bacterial suspension was incubated with lysostaphin in the same conditions to determine the efficiency of bacterial lysis.

Assessment of bacterial killing. Samples (50 μl) of bacteria and cells were taken into 10 ml of ice-cold water. After 1 h the appropriate dilutions were made in water and samples were seeded on to blood-agar plates for colony counts. To determine the initial number of bacteria, the standard sample of bacteria was added to cells that had been cooled at 4°C to inhibit phagocytosis, and then they were put through the standard experimental procedure.

To ensure that the hypotonic lysis of neutrophils released the bacteria and that the reduction in colony counts was not simply a reflection of bacterial aggregation within cells resulting in a reduction in the number of discrete colonies, a comparison was made with another measure of bacterial viability. The cell lysate in distilled water was either plated out and the number of colonies determined, or 100 μl was added to 1 ml of broth containing 3H-glucose 5 μCi. After incubation for 120 min at 37°C in a shaking water bath, the bacteria were centrifuged, washed in isotonic saline until the amount of radioactivity in the washings became constant and the amount of radioactivity incorporated into the bacteria was measured. In eight samples of bacteria that had been incubated with cells and contained a random distribution of residual viable organisms varying between 30 and 100%, there was a strong correlation (r = 0.87, p < 0.001) between the colony counts and the incorporation of radioactivity.

Re-exposure of residual viable bacteria from CGD cells to phagocytosis and killing. Opsonised bacteria (2 × 10⁹) were incubated with cells (2 × 10⁸) from two patients for 16 min and samples were taken to determine phagocytosis and killing. The remaining cells were taken into 50 ml of ice-cold saline containing heparin 5 iu/ml and pelleted by centrifugation at 400 g for 10 min. The cell pellet was resuspended in 1 ml of saline containing lysostaphin 100 μg/ml and heparin 5 iu/ml and incubated at 37°C for 5 min to kill extracellular organisms. The cells were then washed three times in cold RPMI medium at 4°C and containing heparin 5 iu/ml, with centrifugation between washes of 400 g for 5 min at 4°C. The cells were then homogenised in ice-cold water in a small Dounce homogeniser with 20 strokes of a tightly fitting pestle (Kontes Glass Co., Vineland, NJ, USA). Most of the cellular remnants were removed by gentle centrifugation at 500 g for 5 min at 4°C and the bacteria then pelleted by centrifugation at 10 000 g for 10 min. This pellet was then dispersed in RPMI 1640 medium and the particles reopsonised as described above. These particles were then added to a fresh suspension of CGD or normal cells in the standard microbicidal assay described above. Samples were taken at 15 sec and 16 min for assessment of phagocytosis and killing.

Comparison of phagocytosis and killing of dispersed and clumped bacteria. Radiolabelled bacteria from an overnight growth were harvested by centrifugation at 1000 g for 10 min at 4°C. They were then gently resuspended in 2 ml of saline (0.15M containing heparin 5 iu/ml) and the suspension was divided into two portions. One portion was vigorously dispersed by aspiration and expulsion 10 times through a 23-gauge injection needle. Both portions were then opsonised with immunoglobulins as described and centrifuged at 1000 g for 5 min at 4°C. The pellets were washed with 5 ml of cold saline and then gently resuspended in 2.0 ml of cold saline. The dispersed sample was again repeatedly passed through a fine-gauge needle. The size of the bacterial particles was then determined with a Coulter Channelyzer (Coulter Electronics, Cold Harbour Lane, Harpenden, Herts AL5 4UN) which confirmed that the mean particle size of the clumped bacteria was much greater than that of the dispersed organisms. Phagocytosis and killing of the bacteria by neutrophils from a patient with X-linked CGD and a control subject was then determined as described above at 4 min after mixing the organisms with the cells at a concentration of 5 organisms per cell.

RESULTS

Phagocytosis and killing

As shown previously (Segal et al., 1981) phagocytosis by normal cells in this system
was very rapid \((t_1 = 0.65 \text{ min, fig. 1})\) and some of the bacteria re-emerged from the cells after 4 min, whereas phagocytosis by CGD cells was slower \((t_1 = 1.26 \text{ min})\) and bacterial re-emergence was not observed.

Bacterial killing by control and CGD cells was very rapid (fig. 1). When corrections were made for extracellular organisms by regression analysis it could be seen that no organisms remained viable within the normal cells (fig. 2a) and that only 22\% of the intracellular organisms in CGD cells survived (fig. 2b). Regression lines for the normal \((r = 0.89)\) and CGD \((r = 0.74)\) were significantly different from each other \((p < 0.005)\) (Armitage, 1971).

Increasing the ratio of bacteria to cells from 1:1 to 10:1 slightly improved bacterial killing by CGD cells \((p < 0.02, \text{ fig. 2c})\). The addition of methylamine, which elevates the \(pH\) within the phagocytic vacuoles of CGD cells (Segal et al., 1981), also improved bacterial killing (fig. 2d \(p < 0.03\)).
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Bacteria growing in synchronous culture did not appear to be any more or less susceptible to killing by CGD cells at different phases of the growth cycle (fig. 3). In addition, bacteria that survived within CGD cells did not appear to represent a particularly resistant population because they were killed when recovered from within the patient's cells, reopsonised and exposed to fresh normal or CGD cells. Thus in two experiments the mean (± SD) percentages of residual viable bacteria in patients and control cells after incubation for 16 min were respectively 49.0 ± 6.4 and 56.4 ± 3.4, values significantly different from the original numbers of bacteria (p < 0.0025 and < 0.001 by Student's t test) but not from each other. It was impossible to relate killing...
FIG. 3.—Killing by CGD cells of staphylococci at different stages of growth prepared from a synchronous culture. The percentage of intracellular bacteria remaining viable after incubation for 4 min (▪, mean and SE of four cultures) and the number of viable bacteria in the culture (○—○) are shown.

to phagocytosis in this experiment because the viable bacteria represented only a small proportion of the total radioactivity presented to the cell, much of which was in the form of partially processed bacterial material which was poorly opsonised and phagocytosed.

Phagocytosis and killing of clumped and dispersed bacteria

The clumped bacteria represented larger particles than the dispersed organisms (fig. 4). Normal cells phagocytosed the clumped and dispersed cells equally and killed both to the same extent as usual. The patients' cells phagocytosed both groups of organisms normally but killed the clumped organisms less efficiently with the result that the numbers of viable organisms were 2.6 times those obtained with dispersed bacteria.

DISCUSSION

These studies indicate that contrary to common belief (Quie et al., 1967; Mandell and Hook, 1969a and b) neutrophils from subjects with chronic granulomatous disease kill the majority of ingested S. aureus. Why then the discrepancy between the results of these studies and those in the literature? The first reason is almost certainly one of technique. In our studies the bacteria were well opsonised with immunoglobulins and after washing they were mixed with cells in a rapidly stirred chamber. The kinetics of phagocytosis and killing were compared over a short time. In conventional killing assays (Quie et al., 1967; Mandell and Hook, 1969a and b), opsonising serum is added
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Fig. 4.—The profile of size of bacterial aggregates prepared as either dispersed (—) or clumped (-- --) particles.

to the incubation mixture of cells and bacteria, which is mixed less efficiently in rotating test tubes; residual viable bacteria are measured during a period of hours rather than minutes without distinction between intracellular and extracellular organisms. This failure to relate bacterial viability to the extent of phagocytosis is likely to be the most significant technical difference.

A second possible cause of confusion is the way in which results have usually been expressed. The expression of the numbers of residual viable bacteria on a logarithmic scale has had the effect of exaggerating the differences observed between bacterial killing by normal and CGD cells. For example, if the CGD cells kill 80% of the organisms and control cells kill 99.99%, expression of these results on a logarithmic scale indicates 0.7 of a log decrease in the CGD cells as compared with a 4-log reduction in the normals. This obscures the fact that the CGD cells have still killed four out of five of the organisms.

The next subject of these studies related to organisms that were resistant to the microbicidal systems of the CGD neutrophils. This resistance did not appear to be a feature of the bacteria because they were killed equally well at the different phases of
the growth cycle, and the bacteria that survived intracellular killing by CGD cells appeared to be vulnerable when recovered and then re-exposed to a fresh batch of the same cells. Why then should these cells kill only some of the ingested bacteria? Conventional theories propose (see Klebanoff, 1975; Babior, 1978) that the major microbicidal system involves oxygen-dependent free radical and H$_2$O$_2$ generation and that these oxygen radicals are either directly toxic or form H$_2$O$_2$ which acts as the substrate for myeloperoxidase-mediated halogenation of the bacteria. In CGD, this oxidase system is missing (Segal and Jones, 1980), and it has been suggested that cells from these subjects are unable to kill bacteria such as $S$. aureus which contain catalase and fail to generate H$_2$O$_2$, while killing normally those bacteria that generate H$_2$O$_2$, the substrate for their own destruction (Mandell and Hook, 1969b). This theory does not explain how many of the ingested catalase-positive organisms are killed in CGD cells, and by normal cells in anaerobic conditions, or the considerable variability in the efficiency with which these cells kill the different species of catalase-containing organisms (Mandell and Hook, 1969b).

We have recently shown that the oxidase system regulates the pH within the phagocytic vacuoles of neutrophils and monocytes (Segal et al., 1981). In normal cells phagocytosis is followed by a rapid rise in vacuolar pH to 7.8-8 which subsequently falls slowly to reach 6.0-6.5 after about an hour. This initial elevation of pH is not seen in CGD and anaerobic cells, and instead the pH rapidly falls to about 5.5-6.0.

Neutrophils contain a group of cationic proteins that are highly microbicidal in vitro (Odeberg and Olsson, 1975) an effect that is markedly pH dependent, possibly as a consequence of the influence of pH on the binding of these proteins to the bacteria. We believe that the oxidase system may enhance bacterial killing indirectly by optimising conditions within the vacuole for killing by some other mechanism such as the cationic proteins. We suggest that in CGD the normal killing systems function quite well initially and are then arrested by the precipitous fall in pH. In these circumstances, organisms such as staphylococci which grow in clumps might be protected as organisms within the interior of the clump or those that are relatively resistant and not immediately killed might be shielded from the bactericidal components until the effect of these is inactivated by the acid conditions. We attempted to test this hypothesis by measuring the killing of clumped as compared with dispersed bacteria. Although a difficult experiment to perform adequately because the clumped organisms tend to disperse and the dispersed ones to clump, possibly because of agglutinins in the opsonising immunoglobulins, the clumped organisms appeared to be killed equally well by the control cells but less efficiently by CGD cells.

The mechanisms by which the respiratory burst of phagocytes promotes bacterial killing and the reasons for the differing sensitivity of bacteria to killing mechanisms in the presence or absence of oxygen are not entirely clear. The dogma that cells from patients with CGD are unable to kill catalase positive bacteria requires revision.

We thank the Medical Research Council and the Wellcome Trust for support, and Drs R. N. Grüneberg and G. L. Ridgway and the Department of Microbiology at University College Hospital for their helpful co-operation.

REFERENCES

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