Clin. exp. Immunol. (1981) **43**, 201–207.

Defective *Aspergillus* killing by neutrophil leucocytes in a case of systemic aspergillosis

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(Accepted for publication 18 July 1980)

SUMMARY

A persistent defect of *Aspergillus* killing was observed in the neutrophils of a 6-year-old patient with a systemic *A. fumigatus* infection which was highly refractory to anti-mycotic therapy. *Aspergillus* phagocytosis *in vitro* was normal, but nearly 80% of the ingested organisms (versus 30% in the controls) survived intracellularly during the 2-hr assay period. The patient's neutrophils showed a subnormal frequency of nitroblue tetrazolium reduction and a subnormal hexose monophosphate shunt activation in response to phagocytosis. The metabolic responsiveness, however, was clearly superior to that of chronic granulomatous disease neutrophils tested for comparison. The immune status of the patient and the following properties of his neutrophils were found to be normal: random and chemotactic motility, killing of *S. aureus* and *C. albicans*, and the contents of several granula enzymes. Our findings suggest the existence of neutrophil factors or functions which are required for killing *Aspergillus*, but not *S. aureus* and *C. albicans*.

INTRODUCTION

Neutrophils are essential in restraining mycotic infections. In fact, candidiasis and aspergillosis are frequent consequences of defective neutrophil function as in chronic granulomatous disease (CGD) (Johnston & Newman, 1977; Bujak, Kwon-Chung & Chusid, 1974; Hazalum, Anast & Lukens, 1972; Raubitshek *et al.*, 1973; Oh *et al.*, 1969) or myeloperoxidase deficiency (Lehrer & Cline, 1969b). We report here a case of systemic aspergillosis which is characterized by an impairment in the *Aspergillus* killing capacity of the patient's neutrophils. Comparison is made with neutrophils of patients with CGD and mucocutaneous candidiasis.

MATERIALS AND METHODS

Case reports

(1) A.M. is a 6-year-old Italian boy, the only son of unrelated parents from Brescia. Pregnancy, delivery and neonatal development were normal. Varicella at 3 years and parotitis at 5 years were the only infectious episodes prior to a bilateral bronchopneumonia of unknown origin (also at 5 years), which was refractory to antibiotic therapy and resolved very slowly. Immmediately thereafter, A.M. developed systemic aspergillosis with localizations at the nose (a draining abscess), a tibia (oesteomyelitis) and the brain (two abscesses). Biopsies and blood cultures were positive for *Aspergillus fumigatus*. Anti-mycotic therapy (5-fluorocytosine and amphotericin B) resulted in the

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regression of the brain abscesses as shown by computerized axial tomography and the osteomyelitis healed following repeated surgical treatment.

The mother of A.M. has suffered from chronic cutaneous aspergillosis on the back for several years.

During the observation period (September 1978–May 1979), laboratory findings never showed any abnormalities in haematological and immunological parameters: RBC varied between 4·1 to $4{\cdot}7 \times 10^6/mm^3$, haemoglobin 10·9–12·7 g/100 ml, haematocrit 34–38·1, WBC 6,000–10,500/mm^3 (with normal morphology and differentials of 42–54% neutrophils, 42–46% lymphocytes, 2–6% monocytes, 0–4% eosinophils). Immunoglobulin levels (IgG 105–210, IgA 39–141, IgM 122–255 iu/ml) were found to be normal.

Serum anti-*Aspergillus* precipitins were detected by radial immunodiffusion using both standard antigens and an antigenic preparation from the strain isolated from the patient.

Relative and absolute numbers of circulating T and B lymphocytes as well as the lymphocyte proliferative response to phytohaemagglutinin, concanavalin A and pokeweed mitogen were repeatedly within the normal range. Delayed-type hypersensitivity skin tests with PPD, candidin and SKSD were normal. Complement tests included CH50, rabbit erythrocyte lysis, yeast opsonization, bactericidal activity and C3 and C4 determinations; all values were within normal limits.

(2) S.M. is a 4-year-old Italian boy from Turin with chronic granulomatous disease (CGD). His mother is a CGD carrier. Treatment with sulphisoxazole markedly decreased the incidence of infectious episodes, without influencing the *in vitro* defect of neutrophils.

(3) I.L.M. is a 2-year-old girl from Messina with chronic mucocutaneous candidiasis. At her first admission, 1 year ago, she showed cutaneous anergy to candidin and a defect in candidacidal activity in otherwise apparently normal neutrophils (Sacch *et al.*, 1979). Transfer factor therapy partially improved the clinical picture. The candidin test has become positive, but the neutrophil defect has persisted.

Neutrophil function tests. Platelet-free leucocytes were purified from heparinized venous blood by dextran (1%) erythro-precipitation and low-speed spin-washings in phosphate-buffered saline (PBS). Both controls and A.M. leucocyte preparations used were always normal in both yield (*ca* 70%) and differential (monocytes 2–6%).

Normal human serum (a pool of 10 healthy adult volunteers) and patient sera were stored in liquid nitrogen in small aliquots.

Microbicidal activity. This was tested against *Staphylococcus aureus, Candida albicans* and *Aspergillus. S. aureus* (ATCC 6538) was grown in antibiotic medium No. 3 (Difco Laboratories, Detroit) to mid-log phase and washed in fresh medium before use. Bactericidal activity was determined according to Keusch & Douglas (1975) using a bacteria: neutrophil ratio of 1 : 10 which ensured a virtually complete phagocytic uptake of the organisms. Results express the fraction of bacteria killed in 90 min.

Candida albicans, obtained from a hospital source, was maintained on Sabouraud's agar slants and was grown in Sabouraud's broth to log phase before use. Candidacidal activity was assayed as described by Lehrer & Cline (1969a) using a *Candida*: neutrophil ratio of 1 : 1. Results express the fraction of organisms killed in 1 hr.

An analogous test was used for determining the *Aspergillus* killing capacity of neutrophils. In this case, the viability of ingested conidiospores, rather than by supra-vital staining, was assessed by the capacity of live conidia to outgrow a mycelium in appropriate culture conditions. *A. fumigatus* and *A. niger* were obtained from Professor G. Caretta, Institute of Medical Mycology, University of Pavia, and maintained on Sabouraud's agar. Conidia were taken from 72-hr streak-cultures (by touching the surface of the aerial growth with a sterile loop), washed in water and suspended in PBS at a concentration of 5×10^7 conidia/ml; mycelial contamination was minimal and conidiospore viability, as judged by the ability to outgrow into a mycelium in Sabouraud's broth, was over 95%. Five million conidia were added to 1×10^6 neutrophils (both in PBS) in the presence of 50μl of normal human serum in a final volume of 0·5 ml.

After 2 hr at 37°C in a reciprocating shaking bath the neutrophils were lysed by the addition of 10 ml of water. The lysate was vortex-mixed and the conidia were pelleted by centrifugation at 3,000 *g* for 5 min. The pellet was resuspended in 1 ml of Sabouraud's broth and incubated overnight (18

hr) at 18°C. Under these conditions, live conidia form mycelia but do not divide. The cultures were then centrifuged at 3,000 *g* for 5 min and the pellets were resuspended in 0·4 ml of 1% formaldehyde in PBS. The fixed material was viewed by phase-contrast microscopy (×400) and the ratio of live to killed conidia was determined by differential count on 150 organisms.

The interaction of *Aspergillus fumigatus* spores with human leucocytes and serum has been studied by Lehrer & Jan (1970): they found that serum was necessary for optimal phagocytosis, but they did not detect any intracellular killing of the phagocytosed spores. The test we use is very similar to theirs, but we do detect relevant killing of *Aspergillus* spores. The discrepancy might be due to:

(1) Strain differences. In fact their spores were much more resistant than ours to storage (4 weeks at 4°C in Hanks' solution), while ours lose about 50% viability overnight.

(2) Germination conditions. They used a soft agar medium, we use liquid Sabouraud's broth; their conditions might have allowed recovery of damaged spores.

Phagocytosis and nitroblue tetrazolium (NBT) reduction. These were tested essentially as described by Preisig & Hitzig (1971). Suspensions of 10^6 neutrophils in 1·5 ml PBS were dispensed into Nunclon plastic dishes, 4 cm in diameter (Nunc, Denmark) and kept for 30 min at 37°C. After this time, the medium was replaced by 2 ml of PBS, pH 6·8, containing 0·1% NBT and 2×10^7 serum-treated heat-killed yeast cells. After 30 min, the dishes were rinsed with PBS, fixed with methanol and stained with safranine. On each dish, 150 neutrophils were inspected (×1,000, oil immersion) and classified on the basis of the number of ingested particles (0 to 7) and on the presence of formazan deposits (+ or −). These data were used for the calculation of the following functional parameters: *phagocytosis frequency* (number of neutrophils containing particles over total number of neutrophils inspected), *phagocytic index* (average number of particles per neutrophil) and *NBT reduction frequency* (number of neutrophils containing particles and showing formazan deposits over total number of neutrophils containing particles).

Aspergillus conidia phagocytosis was assayed both with this test and with an analogous test in suspension.

Hexose monophosphate shunt (HMP) activity. This was assessed as the production of $^{14}CO_2$ from [1-^{14}C]glucose in the presence of KCN (Keusch *et al.*, 1972). HMP shunt stimulation was tested after phagocytosis of latex (*ca* 50 particles per neutrophil) and of serum-treated yeast (20 particles per neutrophil) and in the presence of H_2O_2, diamide (Kosower *et al.*, 1969) or methylene blue (Cooper *et al.*, 1972). The degree of stimulation obtained is expressed as the quotient of the radioactivity liberated by stimulated neutrophils over that liberated by control neutrophils.

Neutrophil motility was assayed as described by Wilkinson (1977) using Boyden chambers equipped with Millipore filters, pore size 3 μm. Bacterial LPS, 10 μg/ml (Difco Laboratories Detroit), in Hanks' medium containing 5% normal human serum was used as the chemotactic stimulus where appropriate. Both random and directional, i.e. chemotactic, migrations were evaluated at 60 min using the 'leading front' method of Zigmond & Hirsch (1973). These values are expressed as the average distance in μm travelled by the two fastest cells in 10 high-power fields.

Granula enzymes. Granula enzymes were determined in granule extracts obtained from purified neutrophils. Blood samples from the patient and his parents were anticoagulated exactly as described by Bretz & Baggiolini (1974), refrigerated, transported in ice on the same day from Pavia to Berne and processed immediately. The anticoagulated blood was mixed with an equal volume of 2% (w/v) dextran T-500 (Pharmacia Ltd, Uppsala, Sweden) in isotonic saline and the erythrocyte aggregates were allowed to sediment at room temperature. All subsequent steps were made at 4°C. The white supernatant was washed twice by centrifugation at 260 *g* for 5 min and resuspension in saline. The pellet was then resuspended in 5 or 10 ml of 0·225% KCl for 2 min to lyse contaminating erythrocytes and then mixed with twice the volume of 1·8% KCl to restore isotonicity. The cells were homogenized as described by Bretz & Baggiolini (1974) and granules were pelleted by centrifuging post-nuclear supernates at 12,500 *g* for 20 min. They were resuspended in water and used for biochemical assays. Established methods were employed for the determination of peroxidase, *N*-acetyl-β-glucosaminidase, elastase and cathepsin (Bretz & Baggiolini, 1974; Dewald *et al.*, 1975).

Granule pellets from the patient, his parents and unrelated healthy controls were processed for electrophoretic analysis as described by Dewald *et al.* (1975).

Electron microscopy. Electron microscopy was performed on samples from the white cell-enriched supernatant obtained after dextran sedimentation. The specimens were fixed in ice-cold 1·5% glutaraldehyde in 0·05 M sodium cacodylate buffer, pH 7·4, and then processed for cytochemistry and electron microscopy as described by Feigenson & Baggiolini (1978).

RESULTS

Table 1 summarizes the results of most of the functional tests which were performed with the neutrophils of the patient (A.M.) described in this report. The patient's neutrophils were unimpaired in their random and directional migrations, showed a high phagocytic performance as expressed by the frequency of phagocytosing cells and by the phagocytic index, killed normally *S. aureus* and *C. albicans*, but were clearly defective in their microbicidal action on *Aspergillus fumigatus* (and on *A. niger*, data not shown). The defective neutrophils phagocytosed the conidia of *Aspergillus* normally, but most of these survived and were able to develop a mycelium when cultures of neutrophil lysates were made. This was observed when the conidia were phagocytosed both in the presence of sera from normal individuals or from the patient himself. Both parents were *normal* at all neutrophil function tests.

Table 1. Functional test on A.M.'s neutrophils

Tests	No. of expts	A.M.*	Controls†	PTV‡
Chemotactic migration	3	81·83±9·37	98·67(86)	76·84
Random migration	3	27·15±3·95	39·49(87)	11·01
Phagocytosis frequency	3	0·98±0·01	0·96(124)	0·79
Phagocytosis index	3	6·30±0·92	3·28(68)	1·26
Microbicidal activities				
S. aureus	3	0·82±0·05	0·87(80)	0·66
C. albicans	3	0·66±0·13	0·65(35)	0·41
A. fumigatus	6	0·22±0·07	0·69(12)	0·51

* Means and s.d.

† Means; the number of healthy controls is indicated in parentheses.

‡ PTV = pathological threshold value (mean − 1·96 s.d.).

Table 2. Neutrophil microbicidal activities of A.M. in comparison with those of I.L.M. (mucocutaneous candidiasis) and S.M. (CGD)*

Test Organism	A.M.	I.L.M.	S.M.
S. aureus	0·84	0·87	0·12
C. albicans	0·69	0·21	0·08
A. fumigatus	0·18	0·72	0·09

* See Table 1 for normal values.

Table 3. Metabolic activation of neutrophils in response to phagocytosis

Tests*	A.M.	A.M.-M	S.M.	S.M.-M	Controls‡	PTV‡
Frequency of NBT reduction	0·18	0·88	0·01	0·59	0·94(92)	0·80
Relative HMP shunt activities†						
After latex	1·65	4·76	1·10	3·39	5·01(176)	2·92
After yeast	4·60	9·76	1·50	3·80	8·03(12)	4·12

* All data are the mean of two separate experiments (three replicas each).

† $^{14}CO_2$ production from [$1-{}^{14}C$]glucose; values express the ratios between radioactivity liberated by phagocytosing cells and radioactivity liberated by control cells. Experimental variation was $\leqslant 6\%$ (frequency of NBT reduction); $\leqslant 12\%$ and $\leqslant 15\%$ (HMP shunt stimulation after latex and after yeast).

‡ As in Table 1.

In one experiment, the microbicidal activities of A.M.'s neutrophils were compared with those of neutrophils from a patient (Sacchi *et al.*, 1979) with chronic mucocutaneous candidiasis (I.L.M.) and those of neutrophils of a CGD patient (S.M.). Neutrophils from the first two patients killed *S. aureus* normally but differed clearly in their killing activities towards the fungi (Table 2). A.M. killed *Candida* but was almost inactive on *Aspergillus* and the opposite was observed with I.L.M. The neutrophils from the CGD patient were defective at all kinds of killing.

The metabolic response to phagocytosis by the patient's neutrophils and those of reference individuals was tested by measuring NBT reduction or the liberation of $^{14}CO_2$ from [1-^{14}C]glucose. In both tests, A.M.'s neutrophils showed an abnormally low response as compared to neutrophils of normal donors and of A.M.'s parents (the values for A.M.'s mother are shown in Table 3). In the NBT reduction test, only about one-fifth of the patient's neutrophils showed the normal heavy formazan deposits in association with vacuoles, while most of the remaining cells were evenly stained by a fine granular deposit. HMP shunt activation was very low following phagocytosis of latex, but reached about half of the normal value upon phagocytosis of yeast or of *Aspergillus* conidiospores. While being impaired, the metabolic response of A.M.'s neutrophils was clearly superior to that of CGD cells (S.M.). The corresponding values for a clinically healthy CGD carrier (S.M.-mother) are given for comparison (Table 3). Stimulation of the HMP shunt by H_2O_2, diamide or methylene blue was *normal* in all subjects tested, indicating that the impaired responses do not result from enzymatic defects of the shunt or the glutathione cycle.

Peroxidase, *N*-acetyl-β-glucosaminidase, cathepsin B and elastase were all readily detectable in the granule preparations from A.M.'s neutrophils. Since the amount of material available did not permit determination of proteins, comparison with average specific activities (Bretz & Baggiolini, 1974; Dewald *et al.*, 1975) was done on the basis of the original neutrophil count which was 4,800 per μl in the blood sample processed. The proteinase activities were very similar to those of normal neutrophils. *N*-acetyl-β-glucosaminidase was about one-half and peroxidase between one-third and one-quarter of the average activities. In a first sample, electrophoretic analysis of elastase and cathepsin G was unsatisfactory due to insufficient solubilization from the granule pellets. For this reason, fresh blood samples from A.M., his parents and two healthy controls were processed in parallel. In all cases, only faint elastase bands were revealed while cathepsin G was undetectable. However, the electrophoretic patterns obtained from all samples were virtually identical and showed that A.M.'s neutrophils contained normal levels of proteinase 3 (Baggiolini, Bretz & Dewald, 1978) and lysozyme.

Electron microscopy of neutrophils from A.M. and his parents revealed no abnormalities in granule appearance and in the intensity and distribution of peroxidase reaction product. In all three

Table 4. Defect persistence in A.M.'s neutrophils*

Date	Relative HMP shunt stimulation by latex phagocytosis	*Aspergillus* killed *in vitro*
28.9.78	1·65	0·21
2.10.78	1·95	0·32
23.10.78	1·48	0·18
13.11.78	1.50	0·20
4.12.78	2·00	n.t.
29.1.79	1·85	0·12
5.2.79	1·50	0·30
Mean ± s.d.	1·70 ± 0·23	0·22 ± 0·07

* Values are expressed as in Tables 1 and 3, which also contain the normal values. All data are the mean of two separate experiments (three replicas each for HMP shunt stimulation and two replicas each for *Aspergillus* killing). Experimental variation was ≤12% (HMP) and ≤13% (*Aspergillus* killing).

samples, some cytoplasmic extraction was observed, which could have resulted from the more than 12-hr storage period and the subsequent purification procedure.

Anti-mycotic therapy which eventually improved A.M.'s clinical condition failed to influence the neutrophil defect. As shown in Table 4, both the metabolic responses to latex phagocytosis and the *Aspergillus* killing capacity remained virtually unchanged over a 6-month observation period. Under therapy, the killing power of the patient's plasma (diluted 1:2 with PBS) rose considerably from a control value of 0·1 to a maximum of 0·59.

DISCUSSION

An assay for neutrophil microbicidal activity using *Aspergillus* conidia as test organisms has enabled us to define a defect of *Aspergillus* killing in the neutrophils of a patient with chronic systemic aspergillosis. The killing defect was accompanied by an impaired metabolic responsiveness to phagocytosis and by a reduced peroxidase content. The diagnosis of CGD could be ruled out on the basis of the following findings: (1) microbicidal activities against *S. aureus* and *C. albicans* were normal; (2) a partial metabolic response to phagocytosis of opsonized yeast was present; (3) about one-fifth of the neutrophils reduced NBT normally and most of the remainder showed reduction at least to a certain extent; (4) both parents were found to have completely normal neutrophils.

Our investigation failed to uncover functional defects in A.M.'s parents. In particular, the persisting cutaneous aspergillosis of A.M.'s mother does not appear to be associated with a defective killing capacity of her neutrophils.

Several potential microbicidal mechanisms with overlapping spectra of activity are thought to participate in the killing of bacteria and fungi within neutrophils (Klebanoff & Clark, 1978). Most micro-organisms are likely to be susceptible to multiple killing factors, while others appear to require the presence of specific systems. For instance, myeloperoxidase activity is an absolute requirement for the killing of *C. albicans* but not of *C. pseudotropicalis* (Lehrer, 1972). The apparently selective defect of *Aspergillus* killing in the neutrophils of our patient suggests that specific factors (or functions) may be required for the killing of this mycete which are unnecessary for candidacidal and staphylocidal activities. *Aspergillus* killing capacity by the defective neutrophils remains low despite successful chemotherapy. In fact, *Aspergillus* phagocytosis, which is

normal in the patient, may protect the conidia from the action of the anti-mycotic drugs in the plasma.

It is uncertain whether (or how) the defect of *Aspergillus* killing is related to the depressed metabolic response and the reduced peroxidase content of the patient's neutrophils. The proficiency in staphylocidal and candidacidal activities, however, suggests that the patient's neutrophils produce enough superoxide and possess enough peroxidase for most purposes.

We are indebted to Professor G. R. Burgio for his constant interest and advice; to Professor G. Caretta for the blood cultures, the *Aspergillus* strains and the precipitin tests; and to Drs L. Nespoli, R. Maccario and A. Vitiello for the studies on specific immunity. We thank Mr G. Alloni for the excellent technical assistance.

This work was supported in part by a CNR grant to A.G.S.

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Note added in proof

In February 1980, A.M. again developed systemic aspergillosis with lung and brain localizations and was admitted into another hospital. He finally died in July 1980.