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DISORDERS OF NEUTROPHIL FUNCTION

DEFECTS IN THE EARLY STAGES OF THE PHAGOCYTIC PROCESS*

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SUMMARY

Neutrophils from three patients with recurrent infections were studied for defects in the phagocytic process. In one, random and directed migration, adherence and ability to kill Gram-negative bacteria were defective. In a second patient random and directed migration was abnormal, but adherence was unimpaired; this patient also had an impaired ability to kill Gram-negative bacteria. The third patient had defective leucocyte motility, inability to reduce nitro-blue tetrazolium dye, and deficient killing of both *Staphylococcus aureus* and Gram-negative organisms. This patient's mother showed an intermediate bacterial killing defect consistent with the heterozygous state. The previously unrecognized patterns of defects in these patients emphasize the importance of early cell-membrane associated events to the ultimate success of the bactericidal process, and illustrate the heterogeneity of defects responsible for impaired neutrophil function.

INTRODUCTION

Since the description by Bridges *et al.* (1959) of chronic granulomatous disease of childhood, much attention has been paid to syndromes of increased susceptibility to infection associated with defects in the phagocytic process. Much work (Holmes *et al.*, 1967) has suggested that the defect in many of these patients is intimately related to the lysosome associated metabolic events which occur following particle ingestion by neutrophils and macrophages. However, less attention has been paid to dysfunction in earlier stages in the phagocytic process. We have recently studied three patients with chronic and recurring pyogenic disease whose neutrophils display defects which are more closely related to these earlier stages.

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MATERIALS AND METHODS

Leucocyte preparation

Blood was drawn from the antecubital vein into plastic syringes containing 100 units of phenol-free heparin (Lipo-hepin, Riker) per cc blood. One-half volume of 4·5% solution of 500,000 mol. wt dextran (Pharmacia, Sweden) in 0·85% saline was added, and the mixture permitted to settle at 37°C for 30–60 min. The leucocyte-rich plasma was removed and layered on one-half volume of a mixture of 24 vol of 9% Ficoll (Pharmacia, Sweden) and 10 vol of 34% sodium diatrizoate (Hypaque, Winthrop Laboratories) and centrifuged at 400 *g* for 40 min according to the method of Böyum (1968). The supernatant was discarded and the contaminating red cells were lysed with 25 vol of a 0·83% solution of ammonium chloride. The neutrophils were then washed once with 0·85% saline, and diluted in appropriate media. Preparations contained over 98% neutrophils. For experiments involving chemotaxis, random migration, or adherence, where highly purified neutrophil preparations were not required, cells from the cell-rich plasma centrifuged at 150 *g* for 8 min were used.

Random migration

Following a modification of Miller's method (Miller *et al.*, 1971) neutrophils were suspended in a solution of Hanks's buffered salt solution, containing 0·01 mg% bovine serum albumin, to a final concentration of 5×10^6 neutrophils/cc. Heparinized glass capillary tubes (Scientific Products, Evanston) were filled with neutrophils and centrifuged for 2 min at approximately 200 *g*, and then incubated vertically for 4 hr at 37°C. Using an inverted microscope, migration of the leading edge of the packed neutrophil suspension from the underlying red blood cell layer was estimated and marked on the tube with a glass marking pen. The vertical distance between the top of red blood cell layer and the mark (level of neutrophil migration) was carefully measured with a millimeter rule.

Adherence

The method of Spitler *et al.* (1971 abstract, and in preparation) was used.

Chemotaxis

The method of Horwitz & Garrett (1971) was used. Five million neutrophils were diluted in 1 cc of minimal essential medium with 10% fresh human serum, and placed in the upper compartment of a Boyden chamber. The lower portion contained either minimal essential medium, or a filtrate of an *Escherichia coli* culture grown for 24 hr in minimal essential medium. The compartments were separated by a polycarbonate filter (Nuclepore filter, General Electric), with a 3-μm pore size. Chambers were incubated at 37°C for 2 hr, and the filters were then removed, stained, and examined microscopically. A chemotactic index (CI) was calculated by summing the number of neutrophils which had migrated into the filter in five high power fields.

Quantitative nitro-blue tetrazolium dye (NBT) reduction was measured as described by Baehner & Nathan (1968).

Phagocytosis of yeast particles was assayed by the method of Miller (1969). In some cases, additional experiments were performed substituting polystyrene particles (Difco latex, 0·81 μ diameter) for the yeast particles. Quantitative bactericidal assays were performed as previously described (Douglas *et al.*, 1969), as were immunoglobulin and complement levels

(Douglas *et al.*, 1970). A Rebuck skin window was applied as described by Rebuck & Crowley (1955).

Fourteen control subjects of both sexes, ranging in age from 6 months to 52 years were studied, including eight clinically normal individuals, and persons with each of the following diagnoses: pertussis (1), osteomyelitis (1), pneumococcal pneumonia (1), chronic mucocutaneous candidiasis (1), congenital neutropenia (1), benign monoclonal gammopathy (1).

CASE HISTORIES

Case 1. M.K. is a 24-year-old girl of northern European extraction who presented with staphylococcal axillary adenitis 2 years ago. Despite intensive antibiotic and surgical therapy, the adenitis has persisted. Most recent cultures demonstrate *Escherichia coli* and pseudomonas species. She has also had repeated gingival abscesses. The family history was unremarkable.

Case 2. T.C. is a 6-month-old girl of southern Italian ancestry who has suffered four episodes of staphylococcal cervical adenitis. One episode developed while she was receiving intravenous antistaphylococcal antibiotics. Microscopic examination of tissue curretted from one abscess showed giant cells and early granulomatous organization. The family history was unremarkable.

Case 3. A.S. is a 9-year-old Mexican-American boy who has been hospitalized on numerous occasions for pyogenic infections, beginning at the age of 4 months. He has had repeated episodes of pneumonia, and perioral abscesses, and one episode of meningitis in which a Hafnia species was isolated. Candida has been grown from oral surfaces on several occasions.

RESULTS

In all patients immunoglobulin and total haemolytic complement levels were normal or elevated, and the white blood counts and differentials were normal or appropriately elevated with infection. In no cases, at the time of study, were there significant numbers of neutrophils with 'toxic' granulations, nor were there neutrophils less mature than the 'band' form.

Adherence, random migration, and chemotaxis

Results for adherence and random migration for patients and controls are presented in Table 1. Random movement of neutrophils was abnormal in all three patients. Cells from T.C. and A.S. gave similar results whether in their own serum or in normal serum; the patients' serums did not affect normal cell migration. Neutrophil adherence to nylon was abnormal only for M.K. Normal serum failed to correct this defect, while M.K.'s serum had no effect on normal cells.

None of the patients' cells responded normally to the *E. coli* chemotactic stimulus used (Table 2). A Rebuck window was placed on M.K.'s forearm, and changed hourly for 6 hr, and then at 24 hr. No significant cellular response was observed during the first 6 hr, while at 24 hr a moderate neutrophil response was seen.

NBT reduction

Nitro-blue tetrazolium was normally reduced by the cells of M.K. and T.C. following phagocytosis of polystyrene particles, but not by A.S.'s cells (change in optical density = 0·03) (normal controls: 0·11–0·30).

Phagocytosis

Ingestion of yeast particles, and of latex particles, was normal for all three patients' cells

TABLE 1. Neutrophil adherence to nylon and random migration in patients and controls

Patient	Adherence (% cells retained in nylon column)	Random migration (mm migrated by neutrophils in 4 hr)
T.C.	98·4	0
M.K.	66·8	2
A.S.	99	0
Controls	97* (89–100)†	48* (16–70)†

* Mean determination of fourteen controls. † Range.

Bactericidal assays

Leucocytes from M.K. and T.C. were capable of killing strains of *S. aureus* (including that isolated from patient T.C.) at a normal rate. However, their killing of *E. coli* and Serratia marcescens was defective (Fig. 1a). Cells from both of T.C.'s parents killed all three organisms normally.

Leucocytes from A.S. were slow at killing all of the test organisms. Cells from A.S.'s father were entirely normal, but cells from A.S.'s mother showed an intermediate defect (Fig. 1b).

TABLE 2. Chemotactic response to *E. coli* culture filtrate by cells of patients and controls

Patients	Chemotactic index WBC/5HPF entering filter in Boyden chamber	
	Stimulated by *E. coli* filtrate	Unstimulated
M.K.	14	12
Control	155	22
T.C.	9	15
Control	80	5
A.S.	18	21
Control	430	31
A.S. (normal serum)	26	8
Control (A.S. serum)	550	15

TABLE 3. Summary of neutrophil function studies on patients' cells

Patient	Age, sex	Adherence	Random motility	Chemotaxis	Ingestion of yeast	Reduction of nitro-tetrazolium	Bactericidal capacity	Comments
T.C.	6 months Female	↓	↓	↓	N	N	N (*S. aureus*) ↓ (*E. coli, S. marcescens*)	
M.K.	24 yr Female	N	↓	↓	N	N	N (*S. aureus*) ↓ (*E. coli, S. marcescens*)	
A.S.	9 yr Male	N	↓	↓	N	↓	↓ (*S. aureus, E. coli, S. marcescens*)	Mother: partial bactericidal defect Father: normal

↓ = Diminished; N = Normal.

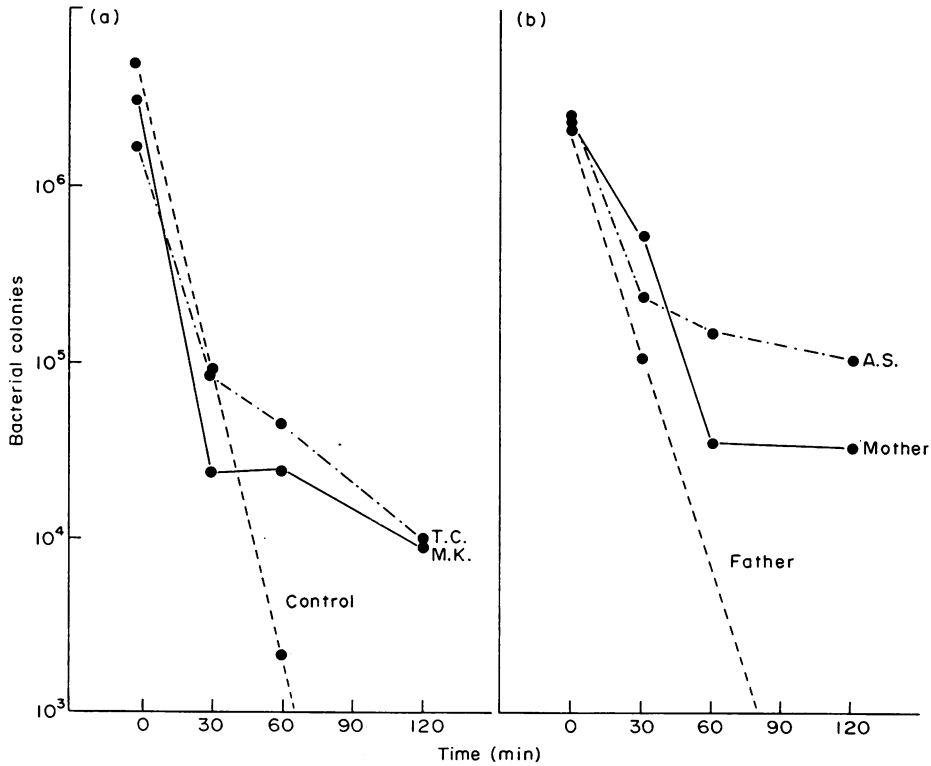

FIG. 1. Bactericidal studies of patients' leucocytes. (a) Impaired killing of *E. coli* by cells from T.C., M.K. Similar results were obtained using *S. marcescens*. (b) Impaired capacity to kill *S. aureus* by cells from A.S. Maternal cells show a lesser defect; paternal cells are normal. Similar results were obtained with *E. coli* and *S. marcescens*.

DISCUSSION

Each of these three patients demonstrates abnormal *in vitro* neutrophil function. *In vivo* function, examined only in M.K., was also abnormal. All three have shown marked susceptibility to infecting bacteria. However, clinical and laboratory aspects of these patients are quite distinct from certain of the better known varieties of phagocytic dysfunction, including the classic chronic granulomatous disease (Holmes *et al.*, 1966). In the latter, the phagocytic dysfunction seems to be at the terminal enzymatic steps responsible for bacteriolysis. However, in order to accomplish the intralysosomal digestion of organisms, several preliminary events must take place, including attachment and opsonization of the particle and lysosomal fusion with the phagosome. Also random, and directed, neutrophil migration (Sorkin *et al.*, 1970) may be necessary to maximize neutrophil–particle interaction. We have identified defects of these early stages in our patients. In all, both random and directed migration were grossly abnormal. In M.K. neutrophil adherence was also severely affected, although opsonization and ingestion of yeast particles proceeded normally. Bactericidal defects could be demonstrated in all three patients, as well as in the mother of one.

Patient A.S. combines defects in cell motility with an inability to reduce nitro-blue tetrazolium dye, and has the characteristic pattern of killing defects in son and mother which have been taken as hallmarks of chronic granulomatous disease (Holmes *et al.*, 1966).

Although clinically and by laboratory study he meets the criteria for the diagnosis of chronic granulomatous disease, he clearly has additional neutrophil defects which are probably not part of this syndrome. Ward and Schlegel, who described a patient similar to A.S. but in whom evidence for a serum inhibitor of neutrophil function was obtained, note that they could demonstrate no defect in neutrophil chemotaxis in boys with chronic granulomatous disease (Ward & Schlegel, 1969). We have studied two additional patients with chronic granulomatous disease who also have no abnormalities in these functions. Such combinations of defects temper the separation of events mediated within the cell from those associated with the cell periphery. Sbarra & Karnovsky (1959) suggest that glycolysis may function as a common energy source for both intracellular killing and for certain surface-associated functions. A defect at this level would explain simultaneous disorders in both systems.

The bactericidal capacity of the cells of A.S.'s mother was intermediate between that of the patient and that of normals. Their other functions were normal, and all studies on paternal cells were normal. A possible interpretation of these results would be that this is a sex-linked disorder, in which the heterozygous carrier may be detected. Less likely is the possibility that the condition is an autosomal dominant with marked sex-limited expression. Similar studies carried out on cells from both T.C.'s parents were normal. M.K.'s parents were not available for study.

Recently, several other patients with neutrophil motility defects have been described, particularly by Miller *et al.* (1971), Alper *et al.* (1970), and Steerman *et al.* (1971). While Alper's patient clearly had a defect related to abnormal complement metabolism, it appears that intrinsic neutrophil defects were present in Miller's patients, and, perhaps in the one reported by Steerman *et al.* (1971) who also had sex-linked agammaglobulinaemia. In none of these patients was cell adherence directly assessed. Miller's patients could be separated into two syndromes. One, apparently sporadic, had markedly diminished random mobility and chemotactic responsiveness. Additionally, a father and daughter were described with defective chemotactic activity, but with normal random migration (Miller & Shonauer, 1971). Bactericidal activity against *S. aureus*, and ability to reduce nitro-blue tetrazolium were normal in both syndromes. Both our patients, M.K. and T.C. are comparable to Miller's patients with a global motility defect, although neither has been neutropenic nor shown any decreased ability to develop a neutrophilia when stimulated by infection. Defective chemotaxis has also been reported as a feature of the Chediak-Higashi syndrome (Clark & Kimball, 1971) though none of our patients showed the other features of this disorder.

The patient described by Steerman *et al.* (1971), combined defects in chemotaxis and phagocytosis with impaired reduction of nitro-blue tetrazolium. He showed an impaired ability to kill staphylococci as a result of his phagocytic defect. As nitro-blue tetrazolium reduction depends on phagocytosis, it is possible that the patient's impaired dye reduction is secondary to diminished phagocytic activity.

Two patients have been described with diminished neutrophil adherence. Higgins *et al.* (1970) reported a 4-year-old Japanese boy with leucocytosis, hypergammaglobulinaemia and recurrent infections. His cells showed normal phagocytic activity, reduction of nitro-blue tetrazolium and killing of *S. aureus*. Neutrophil adherence to glass beads, and migration were defective as in our patient M.K. A 6-year-old girl described by Humbert *et al.* (1970), showed abnormal neutrophil adherence to glass beads, and platelet and red cell membrane functions. White cell phagocytosis and bactericidal activity were normal. Excretion of trimethylamine, a choline metabolite was increased. The relation of this metabolic abnormality to the blood cell membrane defects is unclear.

Our patients, as well as those reviewed above, emphasize the variety of cellular defects which may result in phagocytic dysfunction. Even the phenotype of X-linked chronic granulomatous disease may be associated with more than one underlying defect. They further indicate the importance of events at the cell periphery in maintaining host defences, and emphasize the importance of evaluating these in patients with recurrent infections.

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