Neutrophil cytochemistry in bacterial infection

P. H. MACKIE¹, D. K. MISTRY², J. T. WOZNIAK², W. N. DODDS³,
A. M. GEDDES³, AND J. STUART²

From the ¹Department of Immunology, University of Birmingham B15 2TJ, ²Department of Haematology, Queen Elizabeth Hospital, Birmingham B15 2TH, and ³Infectious Diseases Unit, East Birmingham Hospital, Birmingham B9 5ST, UK

SUMMARY A new cytochemical technique, sensitive to altered lysosomal membrane permeability of blood neutrophils, has been evaluated as a screening test for bacterial infection. This technique, for the lysosomal enzymes acid phosphatase and chloroacetate esterase, was compared with the neutrophil alkaline phosphatase and nitroblue tetrazolium tests. The mean score for each method was significantly higher in infected patients than in normal controls. There was, however, considerable overlap of individual scores between infected patients and ill, but uninfected, patients. This overlap limits the diagnostic value of existing cytochemical screening methods.

It is difficult to obtain rapid laboratory confirmation of a clinical diagnosis of infection, since evidence from bacteriological culture is not usually available for 24 hours or more. There is, therefore, a need for a more rapid screening test in the ill, potentially infected patient.

Patients with bacterial infection commonly have pyrexia, a neutrophil leucocytosis, and an increased erythrocyte sedimentation rate (ESR) but these are nonspecific signs. Their Romanowsky-stained blood neutrophils may, however, show morphological changes (toxic granulation, cytoplasmic vacuolation, decreased nuclear lobulation, and Döhle bodies), which can be of diagnostic value, particularly when studied serially or collectively as a 'degenerative index' (Rosenthal and Sutro, 1933; Meranze *et al.*, 1935). More recently, cytochemical reactions for neutrophil alkaline phosphatase (NAP) activity (Wachstein, 1946; Valentine and Beck, 1951) and nitroblue tetrazolium (NBT) reduction (Park *et al.*, 1968) have been used as screening tests. However, both methods produce false-positive and false-negative results (Hayhoe and Quaglino, 1958; Segal, 1974), and, in one study, the latter method was shown to be no better for diagnosis of bacteraemia than neutrophil number and morphology (Steigbigel *et al.*, 1974).

Toxic granulation results from increased uptake of Romanowsky stain by neutrophil primary lysosomes (McCall *et al.*, 1969), possibly as a result of increased permeability of the lysosomal membrane. A new cytochemical method has shown that addition

of bacteria or their products to a leucocyte suspension *in vitro* causes increased neutrophil membrane permeability (Wozniak *et al.*, 1978). This cytochemical approach, which demonstrates chloroacetate esterase and acid phosphatase activity in primary lysosomes, might form a suitable screening test for bacterial infection. The technique has therefore been studied in parallel with the NAP and NBT tests in 43 patients with bacterial infection, 22 uninfected patients, and 22 normal controls.

Methods

CLINICAL METHODS

Hospital inpatients from an infectious diseases unit and from general medical and surgical wards were studied. The patients with subsequently proved bacterial infection were classified, on the basis of clinical and bacteriological data, as local infection (28 patients) or systemic infection (15 patients). A further 22 inpatients with active disease but no clinical or bacteriological evidence of infection were similarly studied and classified as uninfected patients. Laboratory staff (22 healthy adults) comprised a normal control group. A serial study was also carried out, at the beginning of the trial, of 14 of the patients with bacterial infection (nine localised and five systemic). All laboratory tests were performed without knowledge of clinical details.

Coded blood samples were collected into plastic syringes and transferred to plastic tubes containing heparin (Weddell Pharmaceuticals) to a final concentration of 20 units/ml. All tests were carried out within six hours of sampling.

LABORATORY METHODS

Lysosomal enzyme cytochemistry

The cell suspension technique of Wozniak *et al.* (1978) was used. Chloroacetate esterase and acid phosphatase activities were studied in unfixed neutrophils suspended in a reaction medium of physiological osmolality. This test system gives little or no reaction product for normal neutrophils but results in a coloured deposit when increased lysosomal membrane permeability allows penetration by enzyme substrate. The amount of reaction product was assessed semiquantitatively (Wozniak *et al.*, 1978).

NAP score

The Sigma Chemical Company kit method, based on Ackerman (1962), was used to determine the NAP score in 100 neutrophils on a blood film fixed in 10% v/v formol-methanol at 4°C for 30 seconds.

NBT test

The method of Gordon *et al.* (1975), modified by Wozniak *et al.* (1978), was used.

Statistical methods

Statistical significance was determined by Student's *t* test and correlation coefficient.

Results

SERIAL STUDY

Fourteen infected patients were studied serially. The first sample was taken on day 1 (day of admission, or the following morning if admitted during the night), and further samples were obtained during days 2-4 and 5-14 (see Table). A progressive fall in cytochemical scores occurred in parallel with clinical improvement. All but two patients with a raised NBT score (compared with the normal control group) on days 2-4 also had a raised score on day 1. Only one patient had a raised acid phosphatase score on days 2-4 and a normal score on day 1. With these exceptions, all patients with a raised score on days 2-4 also showed the abnormality on day 1, and all patients with a raised score between days 5 and 14 also had a previously raised score. Subsequent patients were therefore studied on day 1 only.

CHLOROACETATE ESTERASE (Fig. 1)

The mean scores ($\pm$ standard error of the mean) were: normal controls 90.2 ± 2.3 , uninfected patients 97.2 ± 3.2 , patients with localised bacterial infection 106.6 ± 1.5 , and with systemic bacterial infection 117.7 ± 6.7 . There was no significant difference between the mean scores for uninfected patients and

Table Serial study of 14 infected patients showing mean $\pm$ SEM cytochemical scores

	Day 1	Days 2-4	Days 5-14
Number of tests	14	21	29
NBT	36.4 ± 8.9	20.0 ± 5.5	4.2 ± 0.5
NAP	245.6 ± 20.0	241.9 ± 17.9	152.6 ± 17.7
Chloroacetate	104.6 ± 2.2	102.3 ± 1.1	99.7 ± 0.4
Acid phosphatase	3.6 ± 1.9	2.4 ± 0.9	1.4 ± 0.3

Fig. 1 Chloroacetate esterase scores (semiquantitative) for individual patients.

the control group. The mean scores for patients with localised or systemic infection were significantly higher than both the uninfected patient group ($P < 0.01$ and < 0.005 respectively) and the control group ($P < 0.001$).

ACID PHOSPHATASE (Fig. 2)

The mean scores for the uninfected patients (14.6 ± 1.4) and for the systemic bacterial infection group (26.1 ± 8.0) were significantly greater ($P < 0.001$) than for the control group (1.9 ± 0.5). There was no significant elevation of the mean scores for patients with localised or systemic infection compared with uninfected patients.

NAP SCORE (Fig. 3)

The mean scores were: controls 56.5 ± 4.6 , uninfected patients 97.2 ± 5.4 , local bacterial infection 177.5 ± 19.1 , and systemic bacterial infection 192.7 ± 16.5 . Each patient group had a mean score significantly higher ($P < 0.001$) than the

Fig. 2 Acid phosphatase scores (%) for individual patients.

Fig. 3 Neutrophil alkaline phosphatase scores (semi-quantitative) for individual patients.

control group, and patients with localised or systemic infection showed mean scores significantly higher ($P < 0.001$) than uninfected patients.

NBT TEST (Fig. 4)

The mean scores were: controls 3.3 ± 0.4 , uninfected patients 19.9 ± 3.7 , local infection 13.3 ± 1.8 , and systemic infection 43.3 ± 7.2 . The mean score for each patient group was significantly higher ($P < 0.001$) than for the control group. However, only the patients with systemic infection showed a mean score significantly greater ($P < 0.005$) than that of the uninfected patients.

Fig. 4 Nitroblue tetrazolium scores (%) for individual patients.

CORRELATIONS

The chloroacetate esterase and acid phosphatase scores for patients with bacterial infection (localised and systemic infection groups together) correlated with each other ($r = 0.53$, $P < 0.001$); there was no correlation between the other cytochemical scores. Assessment of the four cytochemical tests together, rather than individually, did not result in better discrimination between the infected and non-infected patient groups.

Discussion

The four cytochemical methods used in this study assess different aspects of neutrophil function. The

NBT test depends on the phagocytosis of a tetrazolium-heparin/fibrinogen complex (Segal and Levi, 1973) with reduction of the tetrazole marker; it is thus a test of cytoplasmic membrane activation and phagocytosis (McCall *et al.*, 1974). The chloroacetate and acid phosphatase techniques are sensitive to altered permeability of primary, azurophilic lysosomal membranes of unfixed neutrophils in suspension (Wozniak *et al.*, 1978) whereas the alkaline phosphatase score represents cytoplasmic enzyme activity in air-dried, fixed neutrophils whose cell membranes have been fully labilised (Wozniak *et al.*, 1978). Alkaline phosphatase activity in the mature human neutrophil correlates significantly with cell age (Williams, 1975), being higher in younger cells.

For these tests to be of major clinical value in the diagnosis of infection, they must differentiate not only between infected patients and healthy controls but also between infected and ill but uninfected patients. Each of the cytochemical tests showed a significantly elevated mean score for patients with systemic infection compared with healthy controls. All tests, except acid phosphatase, similarly showed elevation for patients with localised infection. All tests, except acid phosphatase, again showed significant elevation of mean scores for systemic infection compared with uninfected inpatients. There was, however, considerable overlap of individual scores in the different patient groups for all four cytochemical tests. This severely limits the usefulness of neutrophil cytochemical techniques for the diagnosis of infection in an individual patient.

The lysosomal enzyme techniques, when applied to neutrophil concentrates, have proved sensitive to the *in vitro* effects of streptolysin O and phagocytosis of bacteria, in addition to physical agents such as low pH, acetone, and saponin (Wozniak *et al.*, 1978). In some of our infected patients, however, polymorph lysosomal membrane permeability may not have increased sufficiently *in vivo* to be detectable by these techniques; alternatively, neutrophils with fully labilised lysosomal membranes may have been retained at the site of infection and not returned to the systemic circulation. Factors other than infection, such as circulating immune complexes (Henson, 1971), pituitary-adrenal stimulation (Valentine *et al.*, 1954), or tissue damage (Wyllie, 1962), may explain raised scores in uninfected patients.

Existing cytochemical methods, while sensitive to different aspects of neutrophil function, therefore show limitations in the diagnosis of local and systemic infection. Their diagnostic accuracy was not improved by studying four cytochemical tests simultaneously or by a serial study over several days. There are similar limitations for blood culture techniques and tests for circulating endotoxin

(McGill *et al.*, 1970; Martinez-G *et al.*, 1973). The intermittent nature of circulating bacterial products in infected patients, the variable potential for neutrophils to be stimulated at the site of infection and then recirculate, and the loss from the circulation of more extensively damaged neutrophils are variables which increase the difficulty of finding and evaluating diagnostic screening methods based on neutrophil function tests.

We are grateful to the Leukaemia Research Fund and the West Midlands Regional Health Authority for financial support.

References

- Ackerman, G. A. (1962). Substituted naphthol AS phosphate derivatives for the localisation of leukocyte alkaline phosphatase activity. *Laboratory Investigation*, **11**, 563-567.
- Gordon, P. A., Stuart, J., Lee, T. R., Breeze, G. R., and Pugh, R. N. H. (1975). The cytocentrifuge NBT test. *Journal of Clinical Pathology*, **28**, 674-679.
- Hayhoe, F. G. J., and Quaglini, D. (1958). Cytochemical demonstration and measurement of leucocyte alkaline phosphatase activity in normal and pathological states by a modified azo-dye coupling technique. *British Journal of Haematology*, **4**, 375-389.
- Henson, P. M. (1971). The immunologic release of constituents from neutrophil leucocytes. 1. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *Journal of Immunology*, **107**, 1535-1546.
- McCall, C. E., DeChatelet, L. R., Butler, R., and Brown, D. (1974). Enhanced phagocytic capacity. The biologic basis for the elevated histochemical nitroblue tetrazolium reaction. *Journal of Clinical Investigation*, **54**, 1227-1234.
- McCall, C. E., Katayama, I., Cotran, R. S., and Finland, M. (1969). Lysosomal and ultrastructural changes in human 'toxic' neutrophils during bacterial infection. *Journal of Experimental Medicine*, **129**, 267-294.
- McGill, M. W., Porter, P. J., and Kass, E. H. (1970). The use of a bioassay for endotoxin in clinical infections. *Journal of Infectious Diseases*, **121**, 103-112.
- Martinez-G, L. A., Quintiliani, R., and Tilton, R. C. (1973). Clinical experience on the detection of endotoxemia with the Limulus test. *Journal of Infectious Diseases*, **127**, 102-105.
- Meranze, D. R., Mendell, T. H., and Meranze, T. (1935). Cytoplasmic changes in the peripheral neutrophil as an aid in diagnosis and prognosis. *American Journal of the Medical Sciences*, **189**, 639-656.
- Park, B. H., Fikrig, S. M., and Smithwick, E. M. (1968). Infection and nitroblue-tetrazolium reduction by neutrophils: a diagnostic aid. *Lancet*, **2**, 532-534.
- Rosenthal, N., and Sutro, C. J., (1933). The blood picture in pneumonia, with special reference to pathological changes in neutrophils. *American Journal of Clinical Pathology*, **3**, 181-197.

- Segal, A. W. (1974). Nitroblue-tetrazolium tests. *Lancet*, **2**, 1248-1252.
- Segal, A. W., and Levi, A. J. (1973). The mechanism of the entry of dye into neutrophils in the nitroblue tetrazolium (NBT) test. *Clinical Science and Molecular Medicine*, **45**, 817-826.
- Steigbigel, R. T., Johnson, P. K., and Remington, J. S. (1974). The nitroblue tetrazolium reduction test versus conventional hematology in the diagnosis of bacterial infection. *New England Journal of Medicine*, **290**, 235-238.
- Valentine, W. N., and Beck, W. S. (1951). Biochemical studies on leucocytes. *Journal of Laboratory and Clinical Medicine*, **38**, 39-55.
- Valentine, W. N., Follette, J. H., Hardin, E. B., Beck, W. S., and Lawrence, J. S. (1954). Studies on leukocyte alkaline phosphatase activity: relation to 'stress' and pituitary-adrenal activity. *Journal of Laboratory and Clinical Medicine*, **44**, 219-228.
- Wachstein, M. (1946). Alkaline phosphatase activity in normal and abnormal human blood and bone marrow cells. *Journal of Laboratory and Clinical Medicine*, **31**, 1-17.
- Williams, D. M. (1975). Leucocyte alkaline phosphatase as a marker of cell maturity: a quantitative cytochemical and autoradiographic study. *British Journal of Haematology*, **31**, 371-379.
- Wozniak, J. T., Orchard, T. L., Mackie, P. H., Mistry, D. K., and Stuart, J. (1978). Lysosomal enzyme cytochemistry of blood neutrophils. *Journal of Clinical Pathology*, **31**, 648-653.
- Wyllie, R. G. (1962). Neutrophil alkaline phosphatase—response to acute inflammation. *Australasian Annals of Medicine*, **11**, 118-125.
- Requests for reprints to: Dr P. H. Mackie, Department of Haematology, The Queen Elizabeth Hospital, Queen Elizabeth Medical Centre, Edgbaston, Birmingham B15 2TH.