

# Lysosomal enzyme cytochemistry of blood neutrophils

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**SUMMARY** Patients with bacterial infection may show altered membrane permeability of the primary azurophilic lysosomes of blood neutrophils. A new enzyme cytochemical technique, sensitive to increased membrane permeability caused by contact of neutrophils with acetone, saponin, low pH, Streptolysin O, bacteria, and nylon wool, has been developed. The method is of potential value as a screening test for bacterial infection and for detecting neutrophil damage during filtration leucopheresis.

The cytoplasm of the human blood neutrophil is characterised by primary azurophilic lysosomal granules, formed at the promyelocytic stage of differentiation, and secondary specific granules formed at the later, myelocyte stage (Bainton and Farquhar, 1966; Bainton *et al.*, 1971; Bainton, 1975).

The membranes of these lysosomes normally remain impermeable (De Duve, 1969) until the neutrophil exerts its bactericidal role, when lysosomal enzymes are released (Cohn and Hirsch, 1960). Minor damage to lysosomes can, however, cause increased membrane permeability to substances such as enzyme substrate without causing enzyme release (Allison and Mallucci, 1965). The toxic granulation of blood neutrophils in severe bacterial infection probably represents increased permeability of primary lysosomal membranes to Romanowsky stains (McCall *et al.*, 1969) and may either be acquired by the mature neutrophil in the blood (Gordin, 1952; McCall *et al.*, 1973; Wintrobe, 1974) or result from abnormal marrow development or early release (Cline, 1975).

The use of cytochemical methods that are sensitive to altered permeability of primary lysosomal membranes may therefore provide a more sensitive and specific test for bacterial infection than either toxic granulation, the nitroblue tetrazolium (NBT) test, or the neutrophil alkaline phosphatase (NAP) score, which give false positive and negative results (Hayhoe and Quaglino, 1958; Segal, 1974; Steigbigel *et al.*, 1974).

The histochemical technique for acid phosphatase is ideal for testing lysosomal membrane permeability since the  $\beta$ -glycerophosphate substrate does not penetrate the intact membrane of normal cells (De Duve, 1959). Existing cytochemical methods use fixed blood films, however, and fixation itself renders these membranes fully permeable (Allison and Mallucci, 1965). An alternative approach was, therefore, developed, using an unfixed leucocyte concentrate suspended in an incubation solution of physiological osmolality, for both acid phosphatase and a second primary lysosomal enzyme, chloroacetate esterase.

## Cytochemical methods

### LEUCOCYTE-RICH PLASMA

Cytochemical studies were performed on leucocyte-rich plasma (LRP) prepared by sedimenting erythrocytes from 5 ml heparinised blood samples layered on to dextran-Triosil for 30 minutes at room temperature. The dextran-Triosil layer was prepared by mixing 5 ml 3.6% w/v Dextran T500 (Pharmacia) in distilled water with 1 ml Triosil 440 (Nyegaard) in a siliconised glass McCartney bottle and was then sterilised by autoclaving. The LRP layers were aspirated using a plastic pipette and pooled, and 0.5-1 ml aliquots were used for each cytochemical reaction. When five replicate experiments were performed the aliquots were taken from a pool of LRP derived from one individual.

### CHLOROACETATE ESTERASE

The LRP was pipetted into a 5-ml graduated plastic

tube and phosphate buffered saline (PBS) at pH 7.3 was added to 5 ml before centrifugation at 500 *g* for one minute. The supernatant was discarded and the cell pellet resuspended by agitation on a vortex mixer for 5 seconds. Freshly made incubation medium (5 ml) at 37°C was added before incubation in a water bath at 37°C for 10 minutes.

#### Incubation medium

- a Tris-acetate buffer 0.1 M, pH 7.4, containing 1% w/v polyvinylpyrrolidone (PVP), MW 40 000 (Sigma)—25 ml
- b Sodium nitrite solution 4% w/v
- c New fuchsin (Gurr) solution—4% w/v in N HCl
- d Naphthol AS-D chloroacetate (Sigma)—3.0 mg
- e N,N-dimethylformamide (British Drug Houses)—100  $\mu$ l

The new fuchsin solution was hexazotised by mixing with an equal volume of sodium nitrite and the mixture was allowed to stand at room temperature for one minute. The buffer was pre-warmed to 37°C and 250  $\mu$ l of hexazotised new fuchsin solution was added. The substrate was dissolved in the dimethylformamide, added to the buffered fuchsin, mixed, and used immediately. The PVP was added to prevent precipitation of substrate during the reaction. All incubation media used in this study were adjusted to a final osmolality within the physiological range (275-300 mosmol/kg).

After incubation the tube was centrifuged at 500 *g* for one minute, the supernatant was discarded, any residuum being drained on to absorbent paper, and the cells were resuspended by vortex mixing in one drop (approximately 20  $\mu$ l) of 5% Dextran T500 in PBS. One drop of the cell suspension was then smeared on to a glass slide, rapidly dried in warm air, and fixed in formol-acetone (3 ml formalin, 5 ml acetone, and 2 ml water) for 30 seconds at 4°C. The film was counterstained for 60 seconds using Mayer's haematoxylin.

A semiquantitative cytochemical score of the red cytoplasmic reaction product in 100 discrete polymorphs was made as follows:

Grade 0 no colour

- 1 a few cytoplasmic granules only
- 2 many granules with, occasionally, a pale diffuse colour
- 3 deep diffuse colour

The number of cells in each grade was multiplied by the grade number and summed to give a weighted score with a maximum of 300.

#### ACID PHOSPHATASE

This azo dye method was similar to that for chloroacetate esterase except that the incubation period was 60 minutes. The score was expressed as the per-

centage of 100 discrete neutrophils containing a diffuse deep pink reaction product.

#### Incubation medium

- a Acetate buffer 0.1 M, pH 5.6, containing 1% w/v PVP—25 ml
- b Sodium nitrite solution 4% w/v
- c New fuchsin (Gurr) solution—4% w/v in N HCl
- d Naphthol AS-BI phosphoric acid (prepared according to Burstone, 1962)—25 mg
- e N,N-dimethylformamide (BDH)—100  $\mu$ l

#### NITROBLUE TETRAZOLIUM (NBT) TEST

A modification of the method of Gordon *et al.* (1975) was used. Equal volumes (250  $\mu$ l) of LRP and NBT (British Drug Houses) solution (1 mg/ml in PBS) were incubated at 37°C for 10 minutes, and 100  $\mu$ l 15% w/v Dextran T500 (Pharmacia) was added before mixing on a vortex mixer. One drop of the resulting cell suspension was smeared on a glass slide, fixed, and counterstained as in the esterase technique. The percentage of neutrophils containing a clearly visible black formazan deposit was determined by counting 100 discrete cells.

#### NEUTROPHIL ALKALINE PHOSPHATASE (NAP)

The kit method, based on Ackerman (1962), of the Sigma Chemical Company was used to determine the NAP score in 100 neutrophils on a blood film which had been fixed in 10% v/v formol-methanol at 4°C for 30 seconds. The films were then counterstained for 3 minutes with 0.1% aqueous neutral red.

This procedure differed from the primary lysosomal enzyme and NBT methods in that the cells were air-dried and fixed before the cytochemical reaction.

#### Statistical methods

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

#### Results

##### CHOICE OF ANTICOAGULANT

There was no apparent difference in reaction product for the primary lysosomal enzymes using either heparin (Weddel Pharmaceuticals) at a final concentration of 20 iu/ml, dipotassium EDTA 1.5mg/ml, or citrate 0.38% v/v. Mucous heparin was therefore used for all lysosomal studies.

##### EFFECT OF CELL DRYING

When aliquots of LRP were added to the incubation media there was little or no visible reaction end-product, but if the LRP was first smeared on to a

glass slide and air-dried at room temperature for five minutes then a strong reaction product was obtained (Table 1). Smear, dried cells also allowed penetration of trypan blue. The effect of drying, rather than the method of transfer to the slide or the effect of glass contact, appeared to render permeable the cytoplasmic and lysosomal membranes of the cells. Fixation of these air-dried cells in acetone, formol-acetone, or formalin did not result in any further increase in reaction product.

Table 1 *Effect on enzyme scores of cell drying compared with cells maintained in suspension*

	Cytochemical reaction score		
	Chloroacetate esterase	Acid phosphatase	Trypan blue exclusion (%)
Cell suspension	100	0	100
Dried smear on glass	300	100	0
Dried smear on Perspex	300	100	0
Dried cytocentrifuge preparation on glass	300	100	0

The cell suspension technique was therefore adopted for all subsequent studies of primary lysosomal membrane permeability. By comparison, the alkaline phosphatase reaction was performed on air-dried fixed cells with fully permeable membranes.

#### EFFECTS OF PHYSICAL AGENTS

##### *Acetone preincubation*

Aliquots of 0.5-1.0 ml LRP were resuspended in 5.0 ml PBS and acetone was added to give final concentrations of 0%, 1%, 4%, and 8% v/v. After five minutes' exposure to acetone at 37°C the cell suspension was centrifuged at 500 g for one minute and the cell pellet was washed once in PBS before each cytochemical reaction.

The results are shown in Table 2. A 4% final concentration of acetone resulted in a significant increase in activity of acid phosphatase, while both primary lysosomal enzymes showed a significant increase after preincubation in 8% acetone. There was no significant increase in the alkaline phosphatase score of neutrophils with membranes which had previously been rendered fully permeable. Acetone did not increase penetration and reduction of NBT; the NBT score, in fact, decreased slightly.

##### *Saponin preincubation*

A similar experiment was performed using five minutes' preincubation in saponin at concentrations of 0%, 0.001%, and 0.005% w/v.

The results (Table 3) show a significant increase in

Table 2 *Effect of acetone preincubation on enzyme scores (mean  $\pm$  SEM for five experiments)*

	Acetone concentration (v/v)			
	0%	1%	4%	8%
Chloroacetate esterase	103.0 $\pm 1.1$	107.6 $\pm 2.6$	122.6 $\pm 14.2$	144.8 $\pm 12.2$
P		NS	NS	< 0.01
Acid phosphatase	0.6 $\pm 0.2$	1.0 $\pm 0.3$	2.8 $\pm 0.4$	32.2 $\pm 8.4$
P		NS	< 0.005	< 0.01
Neutrophil alkaline phosphatase	83.2 $\pm 1.1$	84.6 $\pm 2.1$	84.8 $\pm 2.6$	85.6 $\pm 2.0$
P		NS	NS	NS
Nitroblue tetrazolium	7.6 $\pm 2.8$	5.4 $\pm 2.2$	3.6 $\pm 1.2$	2.8 $\pm 1.1$
P		NS	< 0.05	< 0.05

Table 3 *Effect of saponin on enzyme scores (mean  $\pm$  SEM for five experiments)*

	Saponin concentration (w/v)		
	0%	0.001%	0.005%
Chloroacetate esterase	104.4 $\pm 0.7$	106.6 $\pm 2.0$	228.2 $\pm 15.9$
P		NS	< 0.001
Acid phosphatase	0.6 $\pm 0.3$	1.6 $\pm 0.5$	93.8 $\pm 2.5$
P		NS	< 0.001
Neutrophil alkaline phosphatase	101.8 $\pm 2.7$	102.4 $\pm 2.2$	100.6 $\pm 3.4$
P		NS	NS
Nitroblue tetrazolium	4.6 $\pm 1.0$	4.4 $\pm 0.9$	3.2 $\pm 0.4$
P		NS	< 0.05

reaction product for both primary lysosomal enzymes at a saponin concentration of 0.005%, but again there was no increase in alkaline phosphatase activity in air-dried neutrophils or in the NBT score.

##### *pH change*

Tris maleate buffer (0.15 M) at pH 4.0, 5.0, 6.0, and 7.0 was adjusted to physiological osmolality with distilled water and warmed to 37°C. The leucocytes from approximately 3 ml LRP were preincubated in the buffer for five minutes at 37°C and then washed, as before, prior to the chloroacetate esterase cytochemical reaction, performed at a pH of 7.4. The results are shown in Table 4. Preincubation at pH 7.0 was taken as the control value, and statistical significance for the other pH values was calculated by comparison. Lowering the pH below 5.0 resulted in a significant increase in cytochemical reaction product.

Table 4 Effect of preincubation at low pH, compared with a control at pH 7.0, on chloroacetate esterase (mean  $\pm$  SEM for five experiments)

	Preincubation pH			
	7.0	6.0	5.0	4.0
Chloroacetate esterase	106.6	105.0	109.4	143.2
P	$\pm 1.9$	$\pm 1.5$ NS	$\pm 3.7$ NS	$\pm 8.6$ <0.005

#### EFFECTS OF BACTERIA AND BACTERIAL PRODUCTS

##### Endotoxin

To 1 ml LRP was added either 1 ml N saline or 1 ml N saline containing 2 mg *Escherichia coli* endotoxin (Difco). Preincubation was carried out for 60 minutes at 37°C. The centrifuged cell pellets were then washed and tested cytochemically as before. For the nitroblue tetrazolium test the cell pellet was instead resuspended in 250  $\mu$ l prewarmed autologous plasma, and an equal volume of prewarmed NBT solution (Gordon *et al.*, 1975) was added before incubation at 37°C for 10 minutes. The suspension was then made up to 5 ml using PBS, centrifuged at 500 g for one minute, and drained. The cell deposit was resuspended in one drop of PBS containing 5% dextran and smears were air-dried on to glass slides. These were fixed in formol-acetone at 4°C for 30 seconds and counterstained with neutral red for three minutes. The percentage of neutrophils containing a definite formazan deposit was counted.

Endotoxin increased the NBT score but had no other significant effect (Table 5).

Table 5 Effect on enzyme scores of preincubation with endotoxin for 60 minutes (mean  $\pm$  SEM for five experiments)

	Control	Endotoxin
Chloroacetate esterase	103.0	99.2
P	$\pm 1.1$	$\pm 2.2$ NS
Acid phosphatase	2.4	2.6
P	$\pm 0.5$	$\pm 0.8$ NS
Neutrophil alkaline phosphatase	62.0	59.4
P	$\pm 3.6$	$\pm 2.0$ NS
Nitroblue tetrazolium	18.0	38.8
P	$\pm 2.5$	$\pm 5.4$ <0.01

##### Streptolysin O

A 1 in 100 dilution of Streptolysin O reagent (Difco) in PBS was made up immediately before use, and 2 ml was preincubated for three minutes at 37°C

with 1-ml aliquots of LRP. As previously, the cells were washed before cytochemical testing.

The results (Table 6) show a significant increase in activity of the primary lysosomal enzymes but no significant increase in NAP or NBT.

Table 6 Effect of Streptolysin O on enzyme scores (mean  $\pm$  SEM for five experiments)

	Control	Streptolysin O
Chloroacetate esterase	105.2	220.8
P	$\pm 1.5$	$\pm 7.0$ <0.001
Acid phosphatase	1.0	89.0
P	$\pm 0.6$	$\pm 2.9$ <0.001
Neutrophil alkaline phosphatase	79.4	76.2
P	$\pm 3.5$	$\pm 4.0$ NS
Nitroblue tetrazolium	6.4	4.0
P	$\pm 1.7$	$\pm 1.4$ NS

##### Phagocytosis of bacteria

Heparinised venous blood (4.5 ml) was incubated for 30 minutes at 37°C with 250  $\mu$ l of a bacterial suspension (final concentration of 10<sup>8</sup> organisms/ml) or latex particles (final concentration of 10<sup>9</sup> particles/ml). A blood film was then made for the NAP reaction, 250  $\mu$ l of blood was taken for the NBT test, and the remaining blood was separated on a dextran-Triosil layer for lysosomal enzyme cytochemistry.

The results (Table 7) show a significant increase in chloroacetate esterase activity after phagocytosis of all three organisms, and in acid phosphatase activity after phagocytosis of two of them. In con-

Table 7 Effect of phagocytosis on enzyme scores (mean  $\pm$  SEM for five experiments)

	Control	Pseudo- monas aeru- ginosa	Staphylo- coccus albus	Candida albicans	Latex particles
Chloroacetate esterase	101.0	117.8	103.6	104.2	100.6
P	$\pm 0.5$	$\pm 4.6$ <0.01	$\pm 0.8$ <0.05	$\pm 0.9$ <0.05	$\pm 0.5$ NS
Acid phosphatase	0.2	16.0	2.8	1.4	1.2
P	$\pm 0.2$	$\pm 2.5$ <0.001	$\pm 0.6$ <0.005	$\pm 0.7$ NS	$\pm 0.6$ NS
Neutrophil alkaline phosphatase	76.4	55.2	74.0	71.2	73.2
P	$\pm 3.2$	$\pm 6.1$ <0.05	$\pm 5.6$ NS	$\pm 1.8$ NS	$\pm 7.7$ NS
Nitroblue tetrazolium	6.6	51.2	47.6	28.6	44.4
P	$\pm 1.0$	$\pm 4.5$ <0.001	$\pm 8.0$ <0.001	$\pm 3.2$ <0.001	$\pm 4.0$ <0.001

Table 8 *Effect of nylon wool contact on enzyme scores (mean  $\pm$  SEM for five experiments)*

	30 minutes' contact		60 minutes' contact	
	Control	Test	Control	Test
Chloroacetate esterase	95.0 $\pm 2.3$	114.2 $\pm 0.6$ <0.001	93.4 $\pm 4.8$	113.8 $\pm 2.7$ <0.01
P				
Acid phosphatase	5.4 $\pm 0.9$	14.4 $\pm 0.9$ <0.001	5.4 $\pm 0.2$	15.6 $\pm 1.2$ <0.001
P				
Neutrophil alkaline phosphatase	16.2 $\pm 2.5$	19.4 $\pm 2.3$ NS	18.0 $\pm 2.3$	19.0 $\pm 2.8$ NS
P				
Nitroblue tetrazolium	19.0 $\pm 1.6$	22.4 $\pm 2.4$ NS	17.2 $\pm 2.6$	23.4 $\pm 3.6$ NS
P				

trast, latex particle ingestion did not increase enzyme scores. The NAP scores did not increase in any experiment, but the NBT scores increased significantly after phagocytosis of either organisms or latex.

#### EFFECT OF NYLON WOOL CONTACT

Aliquots (2.5 ml) of LRP were incubated at 37°C with 150 mg nylon wool fibre (Fenwal Laboratories) in plastic syringes for 30 or 120 minutes. Control syringes, containing LRP but no nylon wool, were incubated under identical conditions. The LRP was then expelled from the syringes, and the cells were washed and tested cytochemically as before. The results (Table 8) show a significant increase in activity of the primary lysosomal enzymes but no significant increase for NAP and NBT.

#### Discussion

The cytochemical technique developed in this study used unfixed cells, suspended in a reaction mixture of physiological osmolality, to avoid gross damage to the neutrophil cytoplasmic membrane, which can affect lysosomes within the cell (Dumonde *et al.*, 1965). Adjustment of osmolality was necessary since isolated primary lysosomes are more susceptible to rupture in hypotonic media than the secondary granules (Bainton, 1973). The duration of incubation was adjusted to give little or no reaction product in intact cells so that any membrane damage would give a visible cytochemical reaction for chloroacetate esterase or acid phosphatase.

In contrast, a conventional NAP reaction was performed on neutrophils in which the lysosomal membranes had already been rendered fully permeable by drying and fixation. Membrane active agents would not therefore be expected to increase NAP

positivity since the cytochemical technique already showed 'maximal' activity. Similarly, the NBT score, which reflects cytoplasmic rather than lysosomal membrane activity, should not be increased by agents which act on lysosomal membranes.

Reduction of pH to below 5.0 is known to labilise lysosomal membranes (De Duve *et al.*, 1955; Chayen *et al.*, 1971), and this was demonstrated for the new technique by incubation at low pH before the chloroacetate esterase reaction, which is performed at pH 7.4. Low concentrations of acetone and saponin, which also increase membrane permeability, caused an increase in demonstrable activity of both chloroacetate esterase and acid phosphatase without increasing either the NAP or NBT reactions.

Although these experiments show an effect on lysosomal membranes it is clearly difficult to exclude a simultaneous effect by an external agent on the cytoplasmic membrane; both membranes presumably became labilised in these experiments. McCall *et al.* (1969) concluded that toxic granulation is an alteration in primary lysosomal membranes and they showed by electron microscopy that the toxic granule is not an autophagic body; but again it was not possible in our experiments to exclude the formation of secondary autophagic lysosomes with consequential increased membrane permeability.

The *in vitro* effects of bacteria and bacterial products are relevant to the diagnostic value of these lysosomal enzyme tests in infected patients. Endotoxin, which stimulates the cytoplasmic membrane to phagocytose and reduce NBT (Park and Good, 1970), did not increase demonstrable lysosomal enzyme activity whereas Streptolysin O, which reacts with membrane lipids to form submicroscopic holes (Peter and Smith, 1977) and causes early lysosomal membrane damage (Hirsch *et al.*, 1963), resulted in a substantial increase. Phagocytosis of latex particles increased the NBT score only, whereas phagocytosis of live organisms increased both the NBT score and primary lysosomal enzyme activity. As expected, the NAP activity in fully labilised lysosomes did not increase.

The cell suspension method does, therefore, appear to reflect increased lysosomal membrane permeability caused by certain physical agents and bacteria or bacterial products. This has been demonstrated for two enzymes but other primary lysosomal enzymes could probably be used since membrane permeability to substrate, rather than the activity of a particular enzyme, is the main determinant of the cytochemical reaction. It might also be of value to develop a similar membrane-sensitive technique for the NAP reaction in secondary lysosomes but the precise intracellular localisation of this enzyme is still under debate.

Preliminary studies (unpublished) in patients with localised and systemic bacterial infection have shown elevated scores for chloroacetate esterase and acid phosphatase. These enzymes should now be studied in parallel with the NAP and NBT reactions to determine whether simultaneous testing of different aspects of neutrophil metabolism in patients with bacterial infection can improve diagnostic precision. The new technique also proved sensitive to contact with nylon wool *in vitro*, and since lysosomal enzyme release has already been used as a marker of cell damage in the preparation of neutrophil concentrates (Klock and Bainton, 1976) the method may also be of value in assessing cell damage during filtration leucopheresis.

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