Myeloperoxidase cytochemistry using 2,7-fluorenediamine

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The myeloperoxidase cytochemical reaction has been well established as a valuable tool in the characterisation of various types of leucocytes. This method helps in the distinction between acute myeloid leukaemias (AML) and acute lymphoblastic leukaemias (ALL) as suggested in the French, American, and British (FAB) classification (Bennett *et al.*, 1976).

The most common substrates used are benzidine derivatives (Graham, 1918; Goodpasture, 1919). We have been using benzidine dihydrochloride (BDC) according to Kaplow's (1965) technique. However, the known carcinogenic potential of these substrates has led to the widespread removal of these reagents for clinical use in the United States, United Kingdom, and Japan.

Inagaki *et al.* (1976) reported the use of the diamine derivative of fluorene: 2,7-fluorenediamine (2,7-FDA) as a good substitute of benzidine for the demonstration of myeloperoxidase in peripheral blood and bone marrow samples.

We have compared qualitatively and quantitatively both reactions in order to assess objectively the usefulness of the new reagent.

Material and method

Freshly made bone-marrow films from 10 cases were used: AML at presentation (6), AML in remission (1), chronic granulocytic leukaemia in blast crisis (CGL-BC) (1), polycythaemia rubra vera (PRV) (1), and ALL (2).

Duplicate films were tested with: (a) the reaction of Kaplow as detailed in Dacie and Lewis (1975), with BDC, and (b) the technique of Inagaki *et al.* (1976) with 2,7-FDA. No loss of enzyme activity was observed when the films were stored unfixed for up to 10 days at 4°C.

The technique of Inagaki *et al.* (1976) was used with slight modifications as follows:

- (a) *Fixative*: 10% formol-ethanol solution.
- (b) *Incubation mixture*: 2,7-FDA (Koch-Light

Laboratories Ltd, Colnbrook, Bucks, England) was used as a hydrogen donor. 40 mg of 2,7-FDA were dissolved in 40 ml of Tris-HCl buffer, pH 8.6, to obtain a saturated solution. After stirring vigorously for five minutes at room temperature, the mixture was filtered to remove the excess of precipitated substrate. Just before use, two drops of 30% H₂O₂ were added to the clear filtrate.

(c) *Counterstain*: 50 ml of Giemsa solution prepared in 1/15 M Sørensen's phosphate buffer (pH 6.8) in the following proportions: 1 drop of Giemsa stain per ml of buffer. The modifications made on the original technique were: (1) *Fixative*: formol-ethanol, instead of copper sulphate or 2.5% glutaraldehyde; (2) *Counterstain*: Giemsa solution at pH 6.8 for 15 minutes instead of Giemsa at pH 6.4 for 10 minutes or Carazzi's haematoxylin. The same fixative and counterstain were used in the BDC technique.

CYTOCHEMICAL REACTION

Films were fixed for one minute, washed in distilled water, and transferred to a coplin jar containing the incubation mixture. After five minutes' incubation at room temperature, they were washed in distilled water for a few seconds, shaken off to remove the excess water, and counterstained in the Giemsa solution for 15 minutes. Finally, they were washed, dried in the air, and mounted using a conventional mounting medium (Diatex, R. A. Lamb). The incubation mixture was stable for more than six weeks when stored at room temperature without the addition of H₂O₂.

Results

QUALITATIVE ASPECTS

With 2,7-FDA, the peroxidase activity was expressed in the positive myeloid cells as yellowish-brown granules in the thin parts of the films and as black or dark brown granules in the thicker parts. All neutrophils and some monocytes showed a distinct fine granulation. Eosinophils and basophils showed large positive granules.

In AML, the positive blast cells had often small or fine granules, and in some cases larger granules along with positive Auer rods were clearly seen (Figure).

The reaction product appeared always stronger when BDC was used as a substrate. However, the morphological recognition of the blasts was easier when 2,7-FDA was used. The reaction with 2,7-FDA was, in general, clean and never overwhelmed the cell surface with precipitates or crystals. In contrast, with the BDC method, formation of precipitates and crystals was often seen.

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Figure Positive myeloperoxidase reaction with 2,7-FDA as substrate in a case of acute myeloid leukaemia (M2). Note a positive Auer rod in two blasts ($\times 1400$).

QUANTITATIVE RESULTS

The percentages of positive cells using both techniques were compared in AML (Table): 500 cells were examined. The percentages of positive blast cells in all the cases showed little difference between the two methods; in cases 4 and 8, there was a higher percentage of positivity with the 2,7-FDA method. In the bone marrow of PRV, the percentage of positive myeloid cells was also very similar with both techniques. In ALL, the reaction was negative in the blast cells.

Discussion

We have shown that the cytochemical method of Inagaki *et al.* (1976) is a good alternative to methods involving the use of benzidine for the demonstration of myeloperoxidase at light microscopy. The objection to the use of benzidine is its carcinogenic potential, and a reliable, safe substitute is required. Although the reaction is generally less strong, it has the advantage that the cells can be seen more clearly because crystals and precipitates do not form as they are apt to do with benzidine. In Inagaki's original paper no comparative study with the classic technique of Kaplow was performed.

2,7-FDA may be a weak carcinogen and has produced breast cancer in rats (Griswold *et al.*, 1966), but it is not registered as a known or suspected

Table Percentage of positive blast cells in AML and CGL-BC

Cas ^a	Classification*	2,7-FDA%	BDC%
1	M1	78	81
2	M4	33.6	35
3**	M2	41	41
4	M4	28	12
5	M1	14	12
6**	M1	90	87
7	M1	1.3	1.5
8	CGL-BC (M5)	10	4

*FAB classification (Bennett *et al.*, 1976)

**Auer rods positive with both techniques

carcinogen by the Department of Health and Social Security. With appropriate laboratory precautions, such as avoiding contact with the skin and avoiding inhalation when handling the dry substance, it is probably safe. 2,7-FDA is not considered an industrial hazard and can be obtained commercially.

A non-carcinogenic substrate, the Hanker-Yates mixture (*p*-phenylene diamine HCl, 1 part; pyrocatechol, 2 parts) has been recommended as an alternative to diamino-benzidine for the demonstration of horseradish peroxidase (Hanker *et al.*, 1977). In our hands, the myeloperoxidase reaction was weak or negative with this reagent when the

other techniques were clearly positive. Even when the reaction was positive, it was not well localised in the cell cytoplasm. Therefore this reagent is of no value in studies of blood and bone-marrow samples.

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Letters to the Editor

Use of low ionic strength salt solution in compatibility testing

There have recently been several publications, starting with that of Löw and Messeter (1974), concerning the use of low ionic strength salt solution (LISS) in antibody identification procedures and when cross-matching (Wicker and Wallas, 1976; Lincoln and Dodd, 1978). Other authors are mentioned in the text below.

In November 1977, we published in our regional booklet 'Technical Notes on Blood Transfusion' details of our own LISS methods and, prompted by the recent publication by Ross and Ducie (1978), we should like briefly to record our findings.

The standard methods of cross-matching that we recommend to hospital blood banks in our region are those described by Tovey and Jenkins (1967) in

the ACP Broadsheet 57. To evaluate the use of LISS in rapid compatibility tests against these standard methods we have examined 100 antibodies of different specificities, as shown in Table 1. The LISS solution used was that described by Moore and Mollison (1976), sterilised and stored at 4°C.

The techniques used were LISS indirect antiglobulin tests with 5-minute, 10-minute, 15-minute, and 20-minute incubation times and an indirect antiglobulin test using physiological (0.14M) saline incubating for 60 minutes. All tests were performed by the slide technique. The results are expressed in Table 2 as the percentage of antibodies giving reactions by LISS antiglobulin tests which are (A) better than, (B) equal to, or (C) not as good as the conventional test. Seven sera did not react as well by the LISS antiglobulin test, using a 10-minute incubation time, as by the conventional antiglobulin test. These sera contained anti-Kell antibodies (3), anti-Fy^a(2), anti-D, and anti-Le^b. In all seven cases the reactions were only marginally weaker by the 10-minute LISS antiglobulin test.

The benefits obtained in increasing the incubation time from 5 to 10 minutes can be clearly seen from the table. Furthermore, two weak anti-Kell sera did not re-

Table 1 Number and specificities of antibodies examined

Anti-D	16	Anti-K	17	Anti-Jk ^a	5
Anti-C	3	Anti-k	2	Anti-Wr ^a	1
Anti-E	13	Anti-Kp ^a	2	Anti-Bg ^a	1
Anti- $\bar{c}$	7			Anti-Kn ^a	1
Anti-e	3	Anti-Le ^a	5	Anti-Lan	1
Anti-S	6	Anti-Le ^b	4	Anti-Vel	1
Anti- $\bar{s}$	2	Anti-Fy ^a	8	Anti-Gy ^a	2

Table 2 Effect of incubation times on performance of antibodies by the LISS antiglobulin test

Order of reaction compared with conventional antiglobulin test	LISS antiglobulin test incubation			
	5 min	10 min	15 min	20 min
Percentage				
A better than	18	28	31	37
B equal to	58	65	66	60
C not as good as	24	7	3	3