

## IMMUNOPHENOTYPING OF PEROXIDASE-NEGATIVE ACUTE LEUKAEMIAS USING IMMUNOALKALINE PHOSPHATASE (APAAP) METHOD

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### Summary

Immunophenotyping of acute leukaemias has become an important diagnostic tool in haematology laboratories as it is now well recognised that the presence of certain surface markers has prognostic significance. In 1988, we experimented with the alkaline phosphatase anti-alkaline phosphatase (APAAP) method for immunophenotyping of leukaemic cells in our laboratory. 48 cases of peroxidase-negative acute leukaemias were studied. Our study showed that 2 peroxidase-negative cases carried myeloid surface markers, 44% were negative for the markers studied and 5% were unclassified due to technical problems. We concluded that the APAAP method is a useful technique for demonstrating cell markers in leukaemic cells as the reaction is reddish and usually intense. We failed to demonstrate surface markers in 44% of the cases probably because of the choice of a limited panel of monoclonal antibodies.

Keywords: Monoclonal antibodies, immunophenotype, leukaemia, immunoalkaline phosphatase, APAAP technique.

### INTRODUCTION

In the early 1970's, the usage of immunocytochemical methods for studying haematological neoplasms resulted in a rapid increase in knowledge of the origin and nature of these disorders. However, owing to the limited availability of satisfactory antisera and indicator reagents, these studies were then confined to very specialised laboratories.

With the advent of monoclonal antisera towards the end of the 1970's, immunocytochemical techniques gained wider use. Even so, the immunocytochemical methods then depended heavily on the availability of suitable fluorochrome conjugates and the immunofluorescent microscope and thus were still not suitable for routine purposes. In the early 1980's, immunocytochemical techniques using enzymes as indicators and light microscopy for viewing were developed. These methods rapidly gained acceptance for routine use in the haematology laboratories!

Immunocytochemistry has been widely used to type acute lymphoblastic leukaemias as the presence of surface markers has been shown to have prognostic significance. For example, children suffering from acute lymphoblastic leukaemias showing CALLA (common ALL) surface markers have the best prognosis and a high cure rate with chemotherapy. In contrast, patients with leukaemic cells showing T or B surface markers have poorer prognosis.<sup>2</sup> Immunochemistry has also been used to

differentiate acute lymphoblastic leukaemias from acute myeloid leukaemias more accurately since the two groups of leukaemias are treated differently. In addition, it has been useful in the demonstration of mixed leukaemias and as a research tool in the understanding of leukaemogenesis.

In 1988, we experimented with these enzymatic immunocytochemical techniques in our laboratory and successfully adapted the alkaline phosphatase anti-alkaline phosphatase (APAAP) method for diagnostic use. In this article, we report our experience with immunophenotyping of peroxidase-negative acute leukaemias, which diagnosis was made on the basis of morphology and cytochemical reactions.

### MATERIALS AND METHODS

#### Subjects

48 consecutive cases of acute leukaemias seen at the Haematology Laboratory, Universiti Kebangsaan Malaysia, classified as peroxidase-negative acute leukaemias by morphology and cytochemical reactions were studied. The ages of the patients ranged from 7 months to 70 years. The study was carried out between July 1988 and December 1989.

#### Samples

Peripheral blood film and bone marrow smears before treatment were processed. If the total white cell count was low, buffy-coat smears were utilised. If it was not possible to stain immediately, the unfixed smears were

wrapped in aluminium foil and stored at **-20°C**. The frozen smears were only removed from the freezer when required, warmed to room temperature and then unwrapped. Smears were fixed in a mixture of methanol and formal acetone just prior to staining.

**Reagents**

The following primary and other mouse monoclonal antibodies from Dakopatts A/S, Denmark were used:

Anti-T	(CD 2)
Anti-B	(CD 22)
hti-CALLA	(CD 10)
Anti-myeloid antigen	(CD 15)
Anti-Megakaryocyte	(CDw 41)
Rabbit anti-mouse IgG (RAM-IgG)	
Mouse-APAAP conjugate	

Other reagents included :

Reagents	Source
Fast Violet B	Difco
Naphthol AS-B1 phosphoric acid	Sigma
Levamisole	Sigma
TRIS	Sigma

**Method**

Immunoenzymatic staining using the APAAP method by a three-stage indirect approach was employed.<sup>1,2</sup> In essence, the fixed smears were incubated in turn with optimally diluted primary monoclonal antibody (1 hour), RAM-IgG (30 minutes) and finally mouse-APAAP (30 minutes) with brief

washings in TRIS-buffer saline between each step. Enhancement (15 minutes) was carried out by repeating the RAM-IgG and mouse-APAAP steps to increase the sensitivity. The alkaline phosphatase reaction was then developed (30 minutes) in a freshly prepared solution using Naphthol AS-B1 phosphoric acid as substrate with Fast violet B salt as the coupling agent. Levamisole, an alkaline phosphatase inhibitor, was added to inhibit endogenous activity. The smears were then counterstained in Harris Hematoxylin and mounted in Apathy's medium.

**Analysis**

The interpretation of staining results was straightforward, as illustrated in Figure 1. Stained slides were examined under light microscopy and compared with positive and negative controls. The positive controls were either peripheral blood from normal individuals or previously positive cases. Negative controls were staining reactions without the primary monoclonal antibodies. A reaction was regarded as positive when reddish staining was observed at the cell surface. 100 blast cells were studied per case and the result was considered positive if more than 10% of these blast cells stained positively.

**RESULTS**

The results are shown in Table 1. There were 5 cases of peroxidase-negative leukaemias that were not classified with certainty due to technical errors where the controls were not working and no further sample was available for testing.



FIG.1: T-Lymphoblasts showing reddish staining for CD 2. APAAP x 400.

TABLE 1

**IMMUNOPHENOTYPE OF PEROXIDASE-NEGATIVE ACUTE LEUKAEMIAS SEEN IN THE HAEMATOLOGY LABORATORY, UNIVERSITI KEBANGSAAN MALAYSIA**

Immunophenotype of blast cells	Positive monoclonal marker(s)	No. of cases
Common ALL	CD 10, CD 22	8
T - ALL	CD 2	5
B - ALL	CD 22	7
Megakaryocytic	CDw41	1
Mixed lineage	CD 2, CD 22, CD15	1
No marker	Negative for above markers	21
Total		43*

\* 5 cases were not classified due to technical problems

**DISCUSSION**

It is interesting to observe that cells classified morphologically and cytochemically as peroxidase-negative lymphoblasts were not necessarily lymphoblasts on the basis of the panel of monoclonal antibodies used in this study. Cases of megakaryocytic or myeloid type would have been missed if the antibodies of these clusters were not incorporated in the panel.

Our study shows that the most common peroxidase-negative leukaemia seen is of the CALLA type. This is similar to the results of other reports.<sup>4</sup> However, 21 cases (44%) show no cell marker. The reason may be 1) the panel has a limited number of monoclonal antibodies, and 2) the monoclonal antibody included may have a short span of reaction for a particular lineage antigen. Besides, CD 15 is not a good overall anti-myeloid antibody because it is negative for the monocytic line. Perhaps different results may be obtained if two or more antibodies for each antigen are used.

The two cases which were positive for anti-myeloid monoclonals (CD 15 and CDw41) were treated as acute myeloid leukemias which carry a grave prognosis. They would not have responded to the standard therapy given to acute lymphoblastic leukaemias. Immunophenotyping had contributed to the management decision.

Since immunophenotyping of peroxidase negative leukaemias has management and prognostic significance, it should be carried out as a complementary test to the morphological and cytochemical studies in all haematology laboratories. Technically, the procedure can be easily carried out as no special equipment is required and all the reagents are available commercially. Although the cost of the various antibodies is currently

rather high, it is hoped that with technological improvements in the production of monoclonal antibodies, together with increasing demands and wider usage, the cost of the antibodies may decrease in the near future.

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