PEROXIDASE ACTIVITY AND NUCLEAR DENSITY ANALYSIS (PANDA) IN THE DIAGNOSIS OF ACUTE PROMYELOCYTIC LEUKAEMIA

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INTRODUCTION

Acute promyelocytic leukaemia (APL) is a subtype of acute myeloid leukaemia (AML), which has both distinct biological and clinical features and is now highly curable\(^1\). APL was first described by Hillestad\(^7\) in 1957 when he reported three patients, which he characterised by ‘a very rapid fatal course of only a few weeks duration’, with a white cell blood picture showing promyelocytes in excess and an allied severe bleeding tendency.

It is characterised by a balanced reciprocal translocation between chromosomes 15 and 17\(^2\), which causes a fusion between the promyelocytic leukaemia (PML) gene and retinoic acid receptor\(\alpha\) (RAR\(\alpha\))\(^3\). There is in effect a maturation arrest at the promyelocyte stage, which is caused by the combination of these two genes and the resulting PML-RAR\(\alpha\) fusion transcript, leading to overall transcriptional suppression\(^4\). Leukaemic promyelocytes accumulate in the bone marrow and peripheral blood and have the unique ability to differentiate, when exposed to retinoic acid and to both differentiate and undergo apoptosis, when exposed to arsenic trioxide (ATO)\(^4\). According to the World Health Organisation (WHO) the annual incidence of APL is 5-8%\(^5\), although in a more recent study\(^4\) this figure is reported to be higher (approximately 10-15%). Although the disease is relatively rare in adults, there is a similar incidence of APL in children with AML in some series, whereas in others,\(^6\) the inclusive percentage was higher compared with adults.

INITIAL APPROACH FOR SUSPECTED APL

Review Morphology

The ostensible diagnosis of APL can usually be made by a review of the peripheral blood smear (PBS) either alone or in combination with the bone marrow aspirate and biopsy by an experienced Biomedical Scientist and/or Haematopathologist usually in alignment with the clinical findings\(^8\). Morphologically the PBS often shows a leucopenia with leukaemic promyelocytes that have abundant irregular-appearing primary azurophilic granules, often with bundles of Auer rods or faggots (Figure 1). Although the nuclear contour is often obscured by the granules it is either typically bilobed or reniform in appearance. This latter feature is of importance in the microgranular variant of APL where the granules are much less prominent giving rise to an almost monoytidoid appearance, which can be diagnostically confusing\(^9\) (Figure 2). Using the French-American-British (FAB)\(^10,11\) morphological classification scheme, a classical APL is referred to as AML M3 and the microgranular variant form as AML M3v. With the 2008 revised WHO classification of myeloid neoplasms and acute leukaemia\(^12\) APL is now classified as AML with t(15;17)(q22;q12); PML-RARA. It is critical that appreciation of these fine nuances of the morphology of APL be detailed by competent practicing Biomedical Scientist/s, as this is the one subtype of AML where early institution of treatment must begin at the first suspicion of the disease and where an experienced Haematopathologist might not be available.

Treatment with all-trans retinoic acid (ATRA) and anthracycline-based chemotherapy induces terminal differentiation of the leukaemic promyelocytes into neutrophils, followed by apoptosis. The combination of ATRA and ATO has transformed a once fatal disease into one that is now curable\(^13\).

Early Death Rate

The propensity to haemorrhage is the striking feature of APL. The prothrombin time (PT) and activated partial thromboplastin (APTT) times can be prolonged including a decrease in the
total fibrinogen level in the majority of cases. This disturbance in coagulation is believed to result from intravascular coagulation initiated by procoagulant found within the granules of the leukaemic promyelocytes. There are also increased levels of fibrinogen-fibrin degradation products, D-dimer and evidence of plasminogen activation, which indicate fibrinolysis and a resulting DIC[13-15].

Premature death is common in APL and is frequently related to the bleeding complications associated with this disorder[16, 17]. Before the introduction of ATRA therapy, early death (ED) related to severe bleeding occurred in up to 26% of cases[18-20]. However, most clinical trials on patients starting treatment with ATRA report ED rates (these are defined as: death during or within seven or thirty days from the start of induction phase chemotherapy or as death during this induction phase) of less than 10%[16, 19, 21-24].

A US population study carried out in 2011[25] reported an overall ED rate of 17.3%. Alarmingly this figure rose significantly in older patients to 24.2% for patients aged ≥55 years compared with 12.3% among those aged ≤34 years, compared to a study published in 2012 on a cohort of 70 patients aged ± 50 years treated for APL at the Stanford Hospital, CA in the US, it was reported that 34% died (19% and 26% died within seven and 30 days of admission respectively)[26]

The conclusion of both studies was to highlight the need to educate all healthcare professionals to recognise the pathogenesis of bleeding complications of APL to ensure the earliest possible treatment, in order to reduce the ED rate and to ultimately improve the overall cure rate.

PEROXIDASE ACTIVITY AND NUCLEAR DENSITY ANALYSIS (PANDA)

Today diagnosis of haematological malignancy is an extremely complex procedure.

It usually includes some, if not all of the following in order to establish a definitive diagnosis: cellular morphology and immunophenotyping (having superseded cytochemistry), cytogenetic, molecular and clinical features.

In the majority of cases an abnormal full blood count (FBC) is the first laboratory indication of a potential case of leukaemia such as APL. Modern 6th generation haematology analysers (e.g. ADVIA 120/2120i, Siemens Healthcare Diagnostics South Africa.) uses a combination of cytochemistry and light scatter measurements to derive the peroxidase activity (PA) and nuclear density (ND) in order to determine the primary white blood cell count (WBC) and the WBC differential respectively (Figure 3).

Peroxidase activity is measured in the peroxidase channel using a heated reaction chamber. The cytochemical reaction is based on two-stage chemistry methodologies that use intracellular myeloperoxidase enzyme to differentiate cells using stain and size characteristics. Red cells are lysed using a surfactant and the remaining white cells are then fixed with formaldehyde. In the presence of hydrogen peroxide and a chromogen 4-chloro-1-napthol, the cells containing myeloperoxidase form a dark precipitate and are further characterised by their light-scatter and light-absorption properties. Neutrophils, monocytes and eosinophils are myeloperoxidase positive whereas lymphocytes and basophils are myeloperoxidase negative[31].

Nuclear density is derived from the basophil/nuclear lobularity channel and provides the primary total white cell count on the ADVIA 120/120i. In a heated reaction chamber phthalic acid lyases the red cells and platelets as well as stripping away the cytoplasm from the white blood cells excluding the basophils. Two-angle light scatter is then used to determine cell size and nuclear density. The BASO cytogram uses this to identify and count the cells and nuclei of each population[31].

When the cytograms from the PA and ND channels are combined (i.e. peroxidase activity and nuclear density analysis (PANDA)) this can be used to look at the shape of the cell populations in order to understand the properties of these cells and to provide information about the presence or absence of leukaemic cells in the peripheral blood.

In a study carried out in 2001[27] the PANDA system was explained in some detail in regard to the pre-classification of a range of leukaemias, which included AML (42 cases were studied). The reported overall accuracy, allowing for some variation
The categories for the PANDA system range from P0 (the absence of myeloperoxidase activity) to P6 as shown in Figure 4 and Table 1:

- **P0** shows the absence of myeloperoxidase activity with no myeloid differentiation. This pattern type is suggestive of total myeloperoxidase (MPO) deficiency and AML subtypes M0, M5a, M6 and M7.

- **P1** demonstrates a scattering at the very top of the main cell cluster. This indicates the presence of a low number of cells with myeloperoxidase activity, with either early or partial myeloid differentiation. This particular pattern is suggestive of AML subtypes M1, M2, M5a.

- **P2** shows a more or less homogeneous cluster of cells. These are separated from the Large Unstained Cell (LUC) area, and they also spread across the monocyte area, and at the beginning of the neutrophil area of the cytogram. This pattern is suggestive of AML subtypes M1, M2, M4, M5a and M5b, and partial MPO deficiency.

- **P3** demonstrates a moderate-to-strong myeloperoxidase activity and a homogeneous cell size. This pattern is suggestive of AML subtypes M1, M2, and M4.

- **P4** demonstrates a strong heterogeneous myeloperoxidase activity and is suggestive of AML subgroups M2, M3v and M4.

- **P5** demonstrates strong myeloperoxidase activity in very large cells and is suggestive of AML subtype M3v.

- **P6** demonstrates extremely high levels of myeloperoxidase activity and is suggestive of AML subgroups M3 and M3v.

### PANDA AND ACUTE PROMYELOCYTIC LEUKAEMIA

A study carried out by the haematology department in Taunton in the U.K., independently validated the previous 2001 study using 140 unselected cases of leukaemia (and some benign conditions). Out of eighteen cases of AML (including one case of APL) the PANDA system achieved a detection accuracy of 94.4%\(^\text{[28]}\). In a study with a slightly larger cohort of patients with AML (including two cases of APL) the overall accuracy was 92%\(^\text{[29]}\).

In order to revalidate the PANDA system for AML a study was carried out in late 2011\(^\text{[30]}\). Its main focus however, was to assess the accuracy and its ability to detect cases of APL. In the 100 cases of AML studied, 11 (11%) were provisionally diagnosed with APL (seven M3 and four M3v). All 11 cases (100%) demonstrated a PANDA profile consistent with the final diagnosis. One case of M3v showed a P4/D1 profile, one M3v a P5/D1 profile, and the remaining nine M3 and M3v cases P6/D1 profiles (Figure 5).

Indeed, APL is the only haematological malignancy allocated to the P6/D1 category\(^\text{[28]}\) and crucially none of the other 89 cases of AML from the above study displayed this characteristic profile.

In conclusion, PANDA does not replace the conventional diagnostic procedures such as assessment of morphology using normal light microscopy, immunophenotyping, molecular and cytogenetic methodologies. It does however, add to these approaches and can provide a crucial early warning that leukemic cells may be present in the peripheral blood.

The P6/D1 PANDA cytogram category is seen exclusively in cases of APL and should 'ring alarm bells' to prompt urgent laboratory and clinical review. Undeniably in some cases of APL with M3 morphology the MPO activity is so intense that the majority of the cells are almost at saturation and 'off scale' on the peroxidase channel cytogram together with the tell-tale D1 nuclear density profile\(^\text{[31]}\).
THE FUTURE

There is no doubt that the US study published this year re-enforced the present ideas about the importance of early diagnosis in APL cases, in order to initiate the earliest possible treatment and possible cure. It also recommended that all clinicians and allied healthcare professionals be aware for this potential diagnosis.

‘As biomedical scientists, we have a responsibility to educate not only our service users, but also each other, for it is through education as much as new technology and new treatments that is the key, for example to improving survival among APL patients’. Finally, the use of pattern-recognition of leukaemic cell cluster distributions on the next generation of laboratory computer systems seems highly likely. The future operators of such systems will not only rely on simple ‘analyser flagging’, but on these computer systems assessing parameters such as the PANDA profiles, to alert those to possible diagnoses for further investigation/s and treatment.

REFERENCES


