INTRODUCTION

Myeloperoxidase (MPO) is an enzyme present in primary granules of myeloid cells and is unequivocal marker of myeloid differentiation which is used routinely in the diagnosis of acute myeloid leukemia (AML) *(Saravanan and Juneja, 2008)*. Reactivity for MPO in 3% or more blasts is the criterion for classifying leukemia as AML. MPO is negative in AML with minimal differentiation, acute monoblastic leukemia and acute megakaryoblastic leukemia *(Miller and Pihan, 2009)*.

Cytogenetic, molecular, cytochemical, and immunophenotypic studies are important in diagnosing AML and defining major subtypes of AML in FAB and WHO classification *(Miller and Pihan, 2009)*. The conventional cytochemical method for MPO is observation under light microscopy. But it requires expertise in cell- morphology recognition, and cannot discriminate minimally differentiated acute leukemia because the cytochemical reaction for MPO is negative *(Tan et al., 2009)*.

The Detection of MPO by flow cytometry had been reported as a rapid and reliable technique for the

diagnosis and classification of acute leukemia. The detection of MPO precursor protein by flow cytometric analysis with monoclonal antibody is essential for the determination of the lineage and precise diagnosis of acute unclassified leukemia *(Leong et al., 2004)*.

Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells by use of a specific antibody, thereby allowing visualization and examination under a microscope. It is a valuable tool for the determination of cellular contents from individual cells. Samples that can be analyzed include blood smears, aspirate, and cytospins *(Lorette, 1999)*. The reason to investigate the presence of MPO by monoclonal antibody is the possibility of detecting the proenzyme form before the enzyme become functional. This may be important for the recognition of poorly differentiated acute leukemia *(Latour et al., 2003)*.

Aim Of The Work

The purpose of this study is to compare between conventional cytochemistry, flow cytometry and immunocytochemistry for detection of MPO reactivity in myeloblasts.

Chapter (1)

ACUTE MYELOID LEUKEMIA

Acute leukemia is a clonal malignant disease of hemopoietic tissue that is characterized by proliferation of abnormal blast cells principally in the bone marrow with impaired production of normal blood cells *(Lichtman and Liesveld, 2006)*.

Acute myeloid leukemia (AML) describes a heterogeneous group of hematological disorders characterized by block in the terminal differentiation of particular hemopoietic cell lineage. It collectively refers to a mixture of distinct diseases that differs as regards their pathogenic evolution, genetic abnormalities, clinical features, response to therapy and prognosis *(Collins, 1995)*.

Cytogenetics and molecular analysis have been instrumental in identifying disease entities among the mixed bag of AML types *(Lowenberg et al., 2003)*.

I. Incidence:

AML is the most common type of leukemia in adults, as it accounts for approximately 25% of all

leukemias in adult in the Western world *(Greenlee et al., 2001).*

It continuously shows 2 peaks in occurrence in early childhood and later adulthood, with an incidence of 3.7 per 100,000 persons and an age-dependent mortality of 2.7 to nearly 18 per 100,000 persons *(Deschler and Lubbert, 2006).*

AML has increasing frequency with age (median 64 years) with incidence 35/100,000 at age 90. It is infrequent in children under 15 years *(Provan et al., 2004)*, as it forms a minor fraction (10-15%) of leukemias in childhood (Fig. 1*)* *(Hoffbrand et al., 2006).*

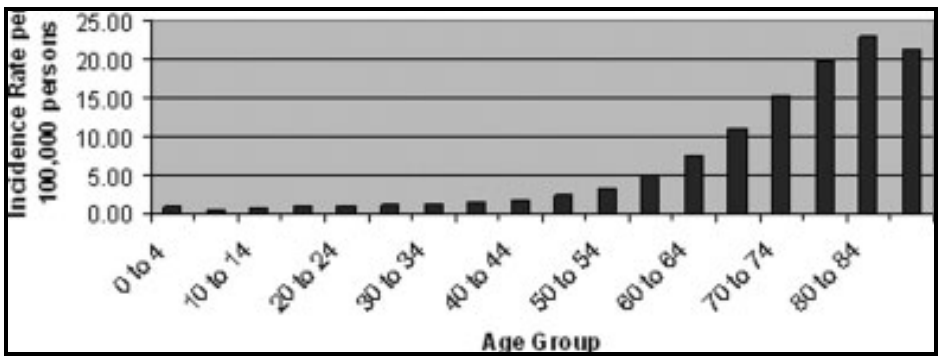

Fig. (1): Age-specific incidence of acute myeloid leukemia in the U.S *(Deschler and Lübbert, 2006).*

In Egypt the overall incidence of acute myeloid leukemia estimated roughly as 3.3 in 100,000 *(www.wrongdiagnosis.com, 2008)*.

II. Predisposing factors:

Many risk factors were found to contribute in the development of leukemia as hereditary and environmental factors *(Wickremasinghe and Hoffbrand, 2000)*.

A. Hereditary factors:

Congenital defect

- Down syndrome
- Bloom syndrome
- Monosmy 7 syndrome
- Kleinfilter syndrome
- Turner syndrome
- Neurofibromatosis

Marrow failure syndrome

- Fanconi anaemia
- Dyskeratosis congenital
- Schwachman - Diamond syndrome
- Amegakaryocytic thrombocytopenia
- Kostman agranulocytosis
- Blackfan diamond syndrome

(Greer et al., 2004)

B. Environmental Factors:

Several agents may contribute to the development of AML:

Solvents:

Benzene its toxicity is related to cumulative dose and leukomogenic risk is considerable at 124 to 200 Part per million (ppm) *(Greer et al., 2004)*.

Smoking:

Smoking has been associated with increased risk of developing AML. It has been reported to be 2 to 3 times higher in male smokers who have exceeded 20 packs per year than non smokers, this could be due to benzene in cigarettes; also tobacco smoke contains potential leukomogenic chemicals including urethane nitrosamine & radioactive compounds *(Greer et al., 2004)*.

Ionizing radiation:

The primary carcinogenic effect of ionizing radiation is causing radiation induced genomic instability in hemopoietic cells which can be shown as non clonal chromosome and chromatid-type aberrations in the clonal progeny of hemopoietic stem cells but the relationship of inducible instability and AML

induction is not known. The same is caused by Gamma radiation *(Gowans et al., 2005)*.

The risk of leukemia correlates with radiation dosage and age at exposure with a more rapid peak early in life as well as more rapid decline than in those exposed at older ages. Atomic tests and exposure to nuclear reactors appear to be an increased risk of leukemia *(Boice and Inskip, 1996)*.

Chemotherapy:

Alkylating agents:

Treatment of patients with lymphoproliferative disorders with alkylating agents as chlorambucil, mustine, melphalan, procarbazine or nitrosourea may predispose to AML especially when these drugs are combined with radiotherapy, the AML patients typically present several years after therapy with peak incidence after about 5 years *(Miller and Daoust, 2000)*.

Topoisomerase II inhibitors:

They are associated with development of AML after a relatively shorter latent period of 2-3 years *(Wickremasinghe and Hoffbrand, 2000).*

C. Acquired diseases:

Certain acquired diseases are associated with transformation to AML as patients with myeloproliferative disorder including polycythemia vera, 1ry thrombocythemia, chronic myeloid leukemia (CML) and agnogenic myeloid metaplasia *(Miller and Daoust, 2000)*.

Aplastic anemia is associated with late development of AML *(Gale et al., 1996)*. In patients with aplastic anemia treated sucessfully with antithymocyte globulin 26% developed AML or one of myelodysplastic syndromes (MDSs) after 8 years & 22% in those who were treated successfully with cyclosporine or recombinant granulocyte Colony stimulating factor (GCSF) *(Miller and Daoust, 2000)*.

AML occurs in patients with paroxysmal nocturnal haemoglobinuira and appears to involve the same clone from which the abnormal erythrocytes are derived *(White et al., 1995)*.

Multiple myeloma is associated with the development of AML. The association of AML, multiple myeloma and administration of multiple alkylating drugs is well documented, but AML can

occur in patients with myeloma who have not received prior chemotherapy or radiation therapy *(Miller and Daoust, 2000)*.

III. Classification:

AML is classified according to morphology, cytochemistry, immunophenotyping (IPT) and cytogenetic features *(Crist and Smithson, 2000)*.

A. FAB classification: (Table 1)

Since 1976, AML has been classified according to the criteria of the French-American-British (FAB) group. This classification is based strictly on morphology and cytochemistry and although it includes two categories linked to chromosomal abnormalities (M3 and M4EO), cytogenetic abnormalities play no part in FAB classification *(Vogler et al., 1992).*

The FAB system does not make provision for the presence of tri or multilineage dysplasia, antecedent chemotherapy or radiotherapy, or preleukemic disorders such as myelodysplasia or myeloproli-ferative disorders (MPDs) *(Vogler et al., 1992).*

Table (1): FAB classification of AML *(Miller and Pihan, 2009)*

FAB subtype (%)	Diagnostic Features
AML-M0 (3%-5%)	≥30% Blasts;<3%blasts reactive to MPO,SBB,or NSE; immunophenotyping CD33+, CD13+ may be CD34+, TdT+
AML-M1 (15%-20%)	≥30% Blasts; ≥3%blasts reactive to MPO or SBB; <10% of marrow cells are promyelocytes or more mature neutrophils
AML-M2 (25%-30%)	≥30% Blasts; ≥3%blasts reactive to MPO or SBB; ≥10% of marrow cells are promyelocytes or more mature neutrophils;t(8:21)chromosome abnormality
AML-M3 (10%-15%)	≥30% Blasts and abnormal promyelocytes; intense MPO and SBB reactivity; promyelocytes and blasts with multiple Auer rods (faggot cells); t(15:17) cytogenetic abnormality
AML-M4 (20%-30%)	≥30% myeloblasts, monoblasts, and promono-cytes; ≥20% monocytic cells in marrow; ≤5×10^9/L monocytic cells in blood: ≥20% neutrophils and precursors in marrow; monocytic cells reactive for NSE;abnormal eosinophils in M4 with associated inv(16) chromosome abnormality
AML-M5a (2%-7%)	≥80% monocytic cells; monoblasts ≥80% of monocytic cells; monoblasts and promonocytes, NSE positive; monoblasts usually MPO and SBB negative
AML-M5b (2%-5%)	≥80% monocytic cells; monoblasts ≥80% of monocytic cells; promonocytes predominate; monoblasts and promonocytes NSE positive; promonocytes may have scattered MPO and SBB positive granules
AML-M6 (3%-5%)	≥50% erythroid precursors: ≥30% of non erythroid precursors are myeloblasts; Auer rods may be present in myeloblasts;dysplastic erythroid precursors frequently are PAS positive
AML-M7 (3%-5%)	≥30%blasts; ≥50% megakaryoblasts by morphology or electron microscopy; immunophenotyping CD41+, CD61+

MPO= myeloperoxidase; SBB= Suddan black B; PAS= Peroidic acid Schiff; CD= cluster designation; NSE= non specific esterase.

B. Cytochemical classification:

Cytochemistry becomes less important than before as a tool in the diagnosis of AML because of the greater efficiency of immunological methods however, MPO and SBB staining are usually indicative of leukemia of myelocytic origin whereas nonspecific esterase (NSE) is indicative of monocytic differentiation. AML blast cells are usually periodic acid Schiff negative with the exception of erythroblasts of M_6 AML and eosinophils of AML of the M_4Eo subclass *(Cheson et al., 1990)*.

C. Immunophenotypic classification (Table 2):

It has been most useful in distinguishing between AML and lymphoid leukemia and in defining hybrid and biphenotypic leukemia *(Catovsky et al., 1991).*

To identify AML, the percentage of positive reacting blasts should be greater than 20% with one or more of the myeloid associated antigens CD33 or CD13 *(Cheson et al., 1990)* . Other myeloid markers as CD11b, CD14, CD15, CD65, CD86, cytoplasmic MPO, CD34 and HLA DR *(Klobusicka et al., 2005)*.

The use of multiple monoclonal antibodies has identified certain phenotypic groups that may be clinically important such as association between M2 subtype with t(8,21) cytogenetic abnormality and the expression of CD34 and the B cell associated cell surface antigen CD19 *(Tisone et al., 1997).*

Table (2): Immunophenotypic markers of AML *(Miller and Daoust, 2000)*

Marker	M0	M1	M2	M3	M4	M5	M6	M7
HLA-DR	++	++	++	-	++	++	+	+
CD11b	+	+	+	-	++	++	-	-
CD13	+	++	++	++	++	++	-	-
CD14	-	+	+	-	++	++	-	-
CD15	-	-	+	+	+	±	-	-
CD33	+	++	+++	+++	+++	+++	+	+
CD41,CD61	-	-	-	-	-	-	-	+++
Glycophorin A	-	-	-	-	-	-	++	-
TDT	++	+	-	-	-	-	-	-
CD34	++	+	-	-	-	-	-	+

It was found that the use of IPT is particularly important for the identification of AML with minimal

differentiation (M_0), erythroleukemia (M_6) and mega-karyocytic leukemia (M_7) (*Miller and Daoust, 2000)*.

Minimal residual disease monitoring in AML by flow cytometry is a promising tool with the potential to significantly improve estimation of prognosis in individual patients. Due to its broad applicability and high sensitivity in minimal residual diseases (MRD) monitoring by multiparameter flow cytometry will serve as a central stratification parameter in future clinical trials *(Kern and Schnittger, 2003)*.

IPT may help in identifying subsets of patients who are at risk for shorter remission duration and resistant disease such as CD 35 positive AML, but the role of IPT as an independent prognostic indicator is unclear *(Tisone et al., 1997).*

D. Cytogenetic classification (WHO classification):

WHO classification attempts to define biological and clinical entities within AML and the relationship between morphology, IPT and genetic abnormalities *(Schoch and Haferlach, 2002)*.

It includes a redefinition of marrow blast percentage and its reduction from 30% to 20% in blood and marrow. Another difference between it and FAB

classification is the categorization of cases of AML into unique clinical and biologic subgroups in the WHO classification. In 2008, a revision of the WHO classification has incorporated recently acquired genetic information into an updated classification scheme of AML (Table 3) ***(Swerdlow et al., 2008).***

Table (3): The 2008 WHO classification system of acute myeloid leukemia ***(Weinberg et al., 2009)***

- **AML with recurrent genetic abnormalities:**
  - AML with t(8;21)(q22;q22); *(RUNX1-RUNX1T1)*
  - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);(CBFB-MYH11)
  - APL with t(15;17)(q22;q12);(PML-RARA)
  - AML with t(9;11)(p22;q23);(MLLT3-MLL)
  - AML with t(6;9)(p23;q34);(DEK-NUP214)
  - AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2);(RPN1-EVI1)
  - AML (megakaryoblastic) with t(1;22)(p13;q13);(RBM15-MKL1)
  - Provisional entity: AML with mutated NPM1
  - Provisional entity: AML with mutated CEBPA
- **AML with myelodysplasia-related changes:**
  - Prior history of myelodysplastic syndrome (MDS)
  - MDS-related cytogenetic abnormality
  - Multilineage dysplasia
- **Therapy-related myeloid neoplasms**
- **Acute myeloid leukemia, not otherwise specified:**
  - AML with minimal differentiation
  - AML without maturation
  - AML with maturation
  - Acute myelomonocytic leukemia
  - Acute monoblastic/monocytic leukemia
  - Acute erythroid leukemia (pure erythroid/erythroleukemia)
  - Acute megakaryoblastic leukemia
  - Acute basophilic leukemia
  - Acute panmyelosis with myelofibrosis
- **Myeloid Sarcoma**
- **Myeloid proliferations related to Down syndrome**
  - Transient abnormal myelopoiesis
  - Other myeloid leukemias of Down syndrome.
- **Blastic plasma cytoid dentrictic cell neoplasm.**