Review Article

The Present Status of Cytochemistry in the Diagnosis of Haematological Malignancy

W. G. STAPLES,   E. P. GÉTAZ

SUMMARY

Newer concepts in the application of cytochemistry to the diagnosis of haematological malignancies are discussed. The underlying physiological principles and location of the various enzymes are indicated. Older stains, once thought to be diagnostic, have been shown to be nonspecific. Newer, more accurate stains are now available.

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The classic morphological methods used to identify the origin of leukaemic blast cells have limitations. Increasing attention has therefore been paid to cytochemical evaluation of metabolic functions and metabolic products. As these techniques have improved, intracellular localization of lipids, glycogen, heparin and various enzymes has become possible. Cytochemistry has demonstrated marked differences in enzyme activity in cells that appear morphologically similar. An accurate diagnosis is mandatory if we are to assess accurately the results of our therapeutic regimens and ultimate prognoses.

At present there are 6 classifications[1-6] in use for the non-Hodgkin's lymphomata and the whole subject has become a matter of acrimonious dispute.[3,4] Histological techniques for the fixation and processing of lymph node sections often produce artefacts.[7] Moreover, techniques vary from laboratory to laboratory, making comparisons difficult and sometimes impossible. For this reason many workers now perform lymph node imprints as a routine and apply cytochemistry for the more precise identification of the malignant cells. Here again comparison of therapeutic modalities depends upon accurate diagnoses.

BIOCHEMISTRY AND PRINCIPLES OF THE STAINING REACTIONS

To understand cytochemical reactions it is necessary to have some knowledge of the function and location of the enzymes and metabolic products involved (Table I). Since the biochemical composition of cells and location of enzymes vary in different cell lines and in the same cell at different stages of maturation, each class of cell

Department of Haematology, Tygerberg Hospital and University of Stellenbosch, Parowvallei, CP

W. G. STAPLES, M.MED. (HAEM. PATH.), F.F.PATH. (S.A.)
E. P. GÉTAZ, M.B. CH.B., M.R.C.P.

will be considered separately. Attention will be directed towards those substances and enzymes which allow cellular identification and it is not proposed that the biochemical reactions of cells be extensively described. Furthermore, an enzyme may be present in more than one class of cell but it will only be discussed if it aids in the identification of a specific cell type, e.g. the acid phosphatase reaction is relevant only in the context of lymphoblastic leukaemia if there is a high percentage of T lymphocytes[8] but it is also present as a lysosomal enzyme in myeloid and monocytic cells. Enzymatic activity is demonstrated by means of specific substrates and appropriate 'couplers' provide a colour localized in the region of enzyme activity. The colour is caused by the union of one of the products of the enzymic action with the coupler.

TABLE I. ENZYMES AND OTHER COMPOUNDS IN CYTOCHEMICAL REACTIONS

Enzymes
  Lysosomal enzymes
    Myeloperoxidase
    Acid phosphatase
    Alkaline phosphatase
    β-glucuronidase
    N-acetyl-β-glucosaminidase
  Enzymes in cytosol
    'Nonspecific esterases'
    Chloro-acetate esterase
Metabolic products
  Glycogen
  Lipid
  Heparin

Granulocytes

Neutrophil precursors contain many mitochondria and respiration is the main source of metabolic energy. As they mature, mitochondria decrease in number and a large depot of glycogen accumulates. Glycolysis is now the predominant mechanism for energy production and the neutrophil can be sustained anaerobically to operate in hypoxic infected tissues and exudates.[9] Glycogen particles accumulate in the metamyelocyte and in band and mature neutrophils which represent the non-dividing non-secretory stages.[10] As glycogen first appears in the myelocyte and increases with maturation, the periodic acid-Schiff (PAS) reaction exhibits progressively increasing positivity. Periodic acid oxidizes the glycol groups in the glycogen to dialdehydes which then react with the Schiff reagent.

The red dyestuff formed by the union of fuchsin-sulphurous acid with dialdehyde is a new compound and not, as previously supposed, re-oxidized fuchsin.[11] Although this is usually a diffuse coloration, in some cases of acute myeloblastic leukaemia it may be in the form of coarse granules.[12]

Myeloblasts are rich in cytoplasmic ribonucleic acid (RNA) engaged in protein synthesis and this accounts for the intense basophilia (blue) with Giemsa stain and the marked pyroninophilia (red) with the methyl green pyronin stain. With maturation the amount of RNA decreases, leading to decreasing basophilia and pyroninophilia. The latter characteristics are important in assessing the degree of maturity of a cell and also its activity in protein synthesis.

Certain enzymes are synthesized in the rough endoplasmic reticulum of promyelocytes and myelocytes and are assembled into granules in the Golgi complex.[10] The primary or azurophilic granules (so named because they are azure after treatment with Wright-Giemsa stains) appear at the promyelocyte stage and constitute primary lysosomes. They contain hydrolytic enzymes which are active during the degradation of phagocytosed material. The enzymes of most importance cytochemically are myeloperoxidase, acid phosphatase, β-glucuronidase and N-acetyl-β-glucosaminidase. Later in development during the myelocyte stage specific granules are produced. These are smaller granules and are specific for neutrophils. The neutrophilic granules contain alkaline phosphatase but lack lysosomal enzymes or peroxidase. Azurophilic granules arise from the concave face of the Golgi complex and specific granules from the convex face. The myelocyte now contains a mixed granule population consisting of smaller pinkish specific granules and large reddish-purple azurophilic granules seen with Giemsa or Wright's stain. With eventual maturation to the neutrophil the cell contains twice as many specific as azurophilic granules.[10]

It is possible to demonstrate peroxidase activity within the entire secretory apparatus of the promyelocyte from the onset of granule production in the endoplasmic reticulum to the Golgi cisternae and azurophil granules. Peroxidases are haemoprotein enzymes and consist of a protein attached to a complex iron-porphyrin prosthetic group. Granulocytes are rich in myeloperoxidase which catalyzes the oxidation of a variety of substances by hydrogen peroxide. After phagocytosis and ingestion of bacteria, fungi and viruses, the azurophil granule or lysosome fuses with the phagosome and hydrolytic enzymes are discharged into it. In the presence of myeloperoxidase and halide ion, molecular oxygen is reduced to hydrogen peroxide, which is one of the most important bactericidal substances.[13]

The principle of the peroxidase reaction is oxidation of benzidine, which yields a blue or brown compound at the site of peroxidase activity. Activity first occurs at the promyelocyte stage and increases to the stage of the mature neutrophil. Eosinophils exhibit a strong reaction which differs from that of neutrophil granulocytes, in that it is cyanide-resistant.[14] Basophils and mast cells are peroxidase-negative.

Alkaline phosphatase can be demonstrated in the secretory apparatus and specific granules of the myelocyte. It was first demonstrated by Roche[15] in 1931 and has subsequently been assayed quantitatively[16] and semiquantitatively by cytochemical means using azo dyes.[17] The substrate used now[12] is naphthol AS phosphate and the coupler is fast blue BBN. Sites of activity are represented by discrete bright blue granules. Characteristically the 'score' is reduced in chronic granulocytic leukaemia for which this stain exhibits absolute specificity.

Granulocytes have considerable amounts of phospholipid concentrated in the membrane of lysosomes which are positive with the lipid stain Sudan black B. The degree of sudanophilia usually parallels the peroxidase reaction. Sometimes the number of positive peroxidase granules is too scant for recognition by light microscopy but Sudan black stains the phospholipid envelope of the granule. Thus Sudan black is a good complementary stain for the peroxidase reaction. Heparin is located in the granules of mast cells and basophils.

Toluidine blue, a metachromatic stain, stains these heparin-containing granules purple to red. Metachromasia signifies the presence of free electro-negative surface charges of a certain minimum density.[11] Both the toluidine blue and peroxidase stains are positive in the rare mast cell leukaemia. These stains become of critical importance in poorly differentiated mast cell leukaemia, where the cells may resemble promyelocytes.[12]

Esterases are cytosol enzymes capable of hydrolysing aliphatic and aromatic esters. Their function in blood is not clear but the chloro-acetate esterase demonstrated in granulocytes may be partially responsible for the debris-digesting action of the neutrophil. Many myeloblasts and all the granulocyte series from the promyelocyte downwards and including mast cells exhibit strong chloro-acetate esterase activity. Basophils exhibit little or no activity and eosinophils are negative. The substrate used is naphthol AS-D chloro-acetate and the coupler is garnet GBC diazo salt; Harris' haematoxylin is used as a counterstain. Positivity is represented by a reddish coloration. The reaction is resistant to sodium fluoride inhibition. Aminocaproate esterase is found in mast cells but cytochemically has no practical value.

Monocytes

These cells, which originate from monoblasts in the bone marrow, migrate into the tissues to become macrophages. Monoblasts have a high content of RNA in their cytoplasm and hence exhibit basophilia but not as intensely as the myeloblast. The neutrophil differs from the mature macrophage in that it is an end cell which terminates with its 'kamikaze'-like phagocytosis. The monocyte actually increases its complement of intracellular organelles as it becomes a macrophage which continues its enzyme synthesis and may live for months in the tissues phagocytosing debris. Monocytes have active aerobic glycolysis[18] and, unlike neutrophils, have little in the way of glycogen stores, so that PAS positivity is slight and variable.

The Golgi apparatus which is small in monocytes increases in size and complexity with cell differentiation to

a macrophage. As in the promyelocyte, azurophil granules or primary lysosomes are synthesized in the rough endoplasmic reticulum and packaged in the Golgi complex. These lysosomes contain similar enzymes to those of the azurophil granules of the myeloid series but continue to be synthesized throughout the life of the macrophage. The monocyte exhibits variable peroxidase positivity for which there is no clear explanation.[19]

There are three cytochemically demonstrable esterases in human blood cells and two have already been discussed, chloro-acetate esterase of granulocytes and aminocaproate esterase of mast cells. The third group is the nonspecific esterases which can be demonstrated in neutrophils and monocytes by means of naphthol AS-D acetate (NASDA) as the substrate and the diazonium salt fast blue BB as the coupler to produce a blue insoluble product. Neutrophils exhibit variable positivity but monocytes show a consistently strong reaction. Specificity is obtained by using sodium fluoride as an inhibitor (NASDA-F). Inhibition occurs only with monocytes.[12]

With alpha-naphthyl acetate esterase as substrate and fast blue B as coupler,[20] monocytes can be identified in smears and histiocytes in lymph node imprints in true histiocytic lymphoma.[2-6] Histiocytic lymphoma is extremely rare and can only be identified positively by this stain.[5] By this means we can differentiate between the histiocyte and the transformed lymphocyte or immunoblast.

N-acetyl-β-glucosaminidase is a lysosomal enzyme that hydrolyses β-glycosidic bonds and releases N-acetyl-β-glucosamine. With naphthol AS-B1-N-acetyl-β-glycosaminide as substrate and fast garnet GBC as coupler, activity can be demonstrated as discrete bluish-purple granules. Monocytes show activity throughout the cytoplasm and over the nucleus and neutrophils may occasionally also exhibit activity.

Histiocytes may contain lipid and will then exhibit a positive reaction with oil red O. Monocytes may also show positivity with Sudan black B. Naturally the degree of positivity with these stains will vary with their lipid content.

Lymphocytes

In the past the lymphocyte was regarded as a completely differentiated cell representing the end stage of lymphoid maturation. As such it may circulate for several years without replicating its deoxyribonucleic acid (DNA) or undergoing mitosis. However, it has been shown that the small lymphocyte is capable of transformation or blastogenesis upon appropriate stimulation.[21] This involves a series of complex biochemical and structural events that causes the lymphocyte to transform into a large basophilic metabolically active cell resembling a blast cell and appropriately called an immunoblast. With blastogenesis the cytoplasm increases in amount and shows intense basophilia due to the increased RNA content. The Golgi apparatus increases in size, the nucleus enlarges and contains prominent nucleoli and the endoplasmic reticulum remains scant. With the immunological characterization of lymphomata and the development of the concept of large-cell lymphomata arising from transformed

lymphocytes,[5] the recognition of these immunoblasts in lymph node imprints assumes great significance. These cells are recognized not only by their morphological features and basophilia on Giemsa-stained sections but also by their pyroninophilia with methyl green pyronin stains.[6] It is as a result of these developments that such terms as immunoblastoma[3] and immunoblastic sarcoma[5] now have real meaning in the pathogenesis of the lymphomata.

Lymph node imprints in lymphoreticular malignancies have been of immense value.[6] Giemsa is the preferred routine stain and identification of prolymphocytes, lymphoblasts and convoluted lymphoblastic cells has been facilitated. Burkitt's tumour has a characteristic appearance, with intensely basophilic vacuolated cells which are pyroninophilic. Lymphoblastic lymphoma[3,4,6,22] has re-emerged in the terminology of the lymphomata, partially as a result of its appearance in lymph node imprints. One of the enigmas in imprints prepared from large cleaved cells on histological sections is that cytologically these cells appear rounded.

Acid phosphatases are lysosomal enzymes found in almost all blood cells. They hydrolyse phosphate esters in an acid environment. Identification is by means of naphthol-AS-B1 phosphate as substrate and pararosanilin hydrochloride as coupler giving rise to bright red granules at sites of activity. T lymphocytes show strong positivity localized to the Golgi region. In cases of T-cell acute lymphoblastic leukaemia more than 80% of cells are positive. As was mentioned in the introduction, the acid phosphatase reaction is of use only in determining the T nature of lymphoblastic leukaemia because the enzyme is also present in myeloid cells.[12]

Tartrate-resistant acid phosphatase is absolutely specific for the rare hairy cell leukaemia. Bennett[12] has found that not all hairy cells show tartrate resistance. Other workers have found tartrate resistance only in hairy cells with strong acid phosphatase activity.[23] In the peripheral blood of patients with T-cell chronic lymphatic leukaemia more than 90% of the lymphocytes show acid phosphatase positivity.

β-Glucuronidase is another lysosomal enzyme present in many cells. It can be identified with naphthol-AS-B1-B-D glucuronide as substrate and hexazonium pararosanilin as coupler.[12] Granulocytes exhibit a diffuse reddish tinge while T lymphocytes show discrete reddish granules in the cytoplasm. Paracortical areas of lymph nodes and peri-arteriolar splenic lymphoid tissue exhibit β-glucuronidase positivity because of their high content of T lymphocytes. It has been claimed that this reaction is more sensitive than the PAS reaction in lymphoblastic leukaemia.[24] β-glucuronidase activity is low in β-cell chronic lymphatic leukaemia and high in the T-cell variety. There is a close correlation between acid phosphatase and β-glucuronidase activity.

For many years the periodic acid-Schiff stain was regarded as important in the differentiation of acute lymphoblastic leukaemias from the other acute leukaemias. Granulocytes show diffuse positivity and 'block' positivity was regarded as specific for lymphoblasts. However, careful

review[12] has shown the PAS reaction to be nonspecific. One-third of all cases of acute myeloblastic leukaemia are PAS-positive, some with coarse granules; nearly 60% of cases of acute myelomonocytic leukaemia are PAS-positive, sometimes with granules; and almost 80% of acute monocytic leukaemias are positive, frequently with 'block' positivity. Therefore nearly half of all cases of acute monocytic leukaemia are peroxidase-negative and PAS-positive, which is the pattern said to be specific for acute lymphoblastic leukaemia.

Oil red O, a diazo dye, selectively stains neutral or simple fats and a positive reaction is due to the presence of the dye within the lipid substance, showing up as round orange-pink areas. Both lymphoid precursors and histiocytes exhibit positivity.

It is important to realize that there is a group of undifferentiated leukaemias which cannot be characterized by conventional means. In a minority of these cases the cells possess markers for normal lymphocytes but as yet their lymphoid nature has not been clearly established.[12] These cells are completely undifferentiated and cytochemistry cannot aid in the diagnosis, since they have not yet developed their normal complement of enzymes and metabolic products. If more than one cell type is present and there are also overlapping groups, diagnosis will depend on the cell type which predominates. This becomes difficult and almost impossible if the marrow sample is hypocellular.

Plasma Cells

These cells contain no free lipids and only small amounts of glycogen and phospholipids. Both respiration and glycolysis are utilized equally. Active protein synthesis continues unabated in mature plasma cells, accounting for their high RNA content with marked basophilia and pyroninophilia.

Megakaryocytes

They contain large amounts of glycogen and their PAS positivity is sometimes useful in differentiating them from bizarre giant cells in tissue sections.

SOME CYTOCHEMICAL STAINS

Bennett[12] has divided cytochemical stains into three groups: (i) cytochemical stains of diagnostic significance — peroxidase, naphthol AS-D acetate esterase without and with fluoride, leucocyte alkaline phosphatase; (ii) specialized cytochemical stains — leucocyte acid phosphatase, naphthol AS-D chloro-acetate esterase, toluidine blue; and (iii) cytochemical stains of limited value — β-glucuronidase, N-acetyl-β-glucosaminidase, periodic acid-Schiff, oil red O, Sudan black B.

It has been shown quite conclusively that the PAS stain is unreliable, and therefore 3 stains are of use in the differentiation of the acute leukaemias, namely peroxidase, and NASDA/NASDA-F esterase (Table II).[12]

TABLE II. CYTOCHEMICAL STAINS AND DIFFERENT FORMS OF LEUKAEMIA

Stains	Acute myeloid leukaemia	Acute myelomonocytic leukaemia	Acute monocytic leukaemia	Acute lymphoblastic leukaemia
Peroxidase	1 - 3+	1 - 3+	0 - 2+	0
NASDA	2+	2 - 3+	3+	0 - 1+
NASDA-F	2+	1 - 2+	0 - 1+	0 - 1+

Peroxidase-positive cells are usually also Sudan black-positive and in rare cases of acute myeloid leukaemia sudanophilia is sometimes more marked than peroxidase positivity. Oil red O is positive with lymphoid precursor cells. Leucocyte alkaline phosphatase remains the specific reaction for chronic granulocytic leukaemia. Acid phosphatase and β-glucuronidase demonstrate T lymphocytes. Toluidine blue exhibits positivity with mast cells. Chloroacetate esterase is used in histological sections to identify leukaemic deposits.

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