The Turkish Journal of Pediatrics 32:135-141, 1990

CELL CYCLE ANALYSIS OF NORMAL AND MALIGNANT LYMPHOID CELLS*

*Dario Campana MD***, George Janossy MD****

Various stages of lymphoid differentiation in the bone marrow (BM) and in the thymus are defined with combinations of monoclonal antibodies (McAbs) and heterologous antisera. The analysis of the proliferative capacity of these cell types can also be performed. One method is the separation of subsets of cells by fluorescent activated cell sorting or panning techniques followed by ^{3}H–thymidine incorporation and cell cycle analysis using propidium iodide[1]. Recently, the availability of McAbs to 5'bromo-2'deoxyuridine (BrdU), a thymidine analogue[2], and to Ki67, a nuclear antigen expressed by cells in cycle but not by cells in Go[3], has facilitated the immunological characterization of dividing cells. Double colour immunohistochemistry and double colour immunofluorescence methods including these Abs have been reported[4,5].

We have studied the proliferating capacity of immature lymphoid cells using multiple marker analysis. Our aim has been to combine the identification of surface, cytoplasmic and nuclear antigens with staining using BrdU and Ki67 McAbs. For this purpose cytocentrifuge preparations are fixed in cold methanol for 30 minutes, followed by immersion in $1x10^{-2}$ N NaOH for 10 seconds and in borate buffer for 5–10 seconds. Fixation in methanol alone, without immersion in NaOH, is optimal for Ki67 staining. With these methods, BrdU and Ki67 staining can now be included in triple colour labelling using specific second layers conjugated to fluorescein isothyocyanate (FITC), tetramethyl rhodamine (TRITC) and colloidal gold[6].

Phenotypic expression and proliferative activity during BM B cell differentiation

The proliferative activity and the differentiation process in the central lymphoid organs of mice have been extensively studied. The BM is a major source of new lymphocytes, and pre-B cells generate a number of small lymphocytes in the order of 10^{8} cells/day. Large cytoplasmic μ^{+}, surface μ^{-} ($c\mu^{+}$, $s\mu^{-}$) cells are the major proliferative compartment in the early stages of B cell differentiation[7,8] reviewed in 9.

* From the Department of Immunology, Royal Free Hospital Medical School, London, England.
** Associate Professor of Immunology, Royal Free Hospital Medical School.
*** Professor of Immunology, Royal Free Hospital Medical School.

In man, the phenotypic changes of lymphoid populations in the BM have also been described. In human fetal BM, around the 17th gestational week, IgM^+ B cells represent 20-25% of all nucleated cells[10]. Relatively high percentages of early B cells express nuclear terminal deoxynucleotidyl transferase (TdT) and/or cμ in fetal and infant as compared to normal adult BM[10-13].

Early studies of the antigenic expression of B cell precursors in the human BM performed with heterologous antisera showed that TdT^+ cells are $HLA\text{-}DR^+$ and express the cALL antigen[14]. These findings have been confirmed and extended with other McAbs against B cell associated antigens. The conclusions of these investigations are as follows: Firstly, phenotypical differences can be found between B cells in the BM and in the peripheral lymphoid organs. On one hand, some antigens, like those identified by CD19[15] and anti Class II reagents, are expressed by virtually all B cells until they become plasma cells. On the other hand, some antigens are associated with particular stages of differentiation. Secondly, there is an orderly appearance of nuclear, cytoplasmic and surface antigens during B cell differentiation. Finally, leukaemic cells of the B lineage reproduce the phenotypic features found on the equivalent normal cells.

TdT^+ cells are Class II^+ and express proliferation-activation related structures such as transferrin receptors and Ca^{++} channels[11,12,16]. Additionally, they express a number of molecules whose function has not yet been fully elucidated. These are the antigens identified by CD10, CD19, CD24 and CD9 antibodies. While CD24 and CD9 are also expressed by non-B cells, CD19 appears to be exclusively B cell associated. CD10 is also found on non-B cells such as granulocytes and some T acute lymphoblastic leukaemia (T-ALL) blasts. Normal BM TdT^+ cells do not express IL2 receptors, but these can be induced by stimulation with phorbol esters (Campana D, unpublished observations).

The phenotype of pre-B cells, identified by the presence of cμ, represents a step forward in the B cell differentiation pathway[17,18]. Nuclear TdT is expressed only by a minority of these cells[12]. The initiation of the synthesis of structures for antigen recognition and the detection of these molecules in the cytoplasm of B cell precursors precedes the expression of surface CD20 and human leucocyte function associated (HLFA) antigens[19-21].

The pattern of early assembly followed by the surface insertion is not only found for Ig expression but also for the structures identified by CD22 McAbs. These molecules are present in the cytoplasm first and absent on the surface of TdT^+ and pre B cells. Subsequently at a later stage of differentiation CD22 is expressed on the B cell membrane[19].

The transition from pre-B cells to $sIgM^+$ lymphocytes is characterized by the progressive acquisition of CD37 and unclustered surface antigens such as RFB7 and Y29/55[19,20,22]. However, the complete set of surface antigens found on

mature B cells in peripheral lymphoid organs is not yet exhibited on these cells, and a number of structures such as the C3d receptor (CD21) and CD22 are absent. Recently, a functional role for some of these B cell associated molecules has been postulated. McAbs of the CD19, CD20 and CD22 clusters have been implicated in the control of B cell proliferation after stimulation with anti-IgM McAbs[23-25]. Complement receptors have also been indicated as important structures in the proliferation of B cells after antigen stimulation. Thus, the 'incomplete' phenotype of IgM^+, IgD^- B cells is in line with their functional 'immaturity'[26].

We have recently documented the proliferative activity of these different subpopulations of B cell precursors in man. We studied the incorporation of BrdU and the expression of nuclear Ki67 of B cell precursors in adult as well as infant and regenerating BM. In order to precisely identify cell subsets, combinations including BrdU or Ki67 with nuclear TdT, surface CD10, cytoplasmic IgM and surface RFB7 (CD20-like) were used.

The results of these studies indicate that during B cell ontogeny in the BM the major proliferative compartments are represented by TdT^+ cells and $c\mu^+$ pre-B cells. About 40 % of TdT^+ cells express Ki67 and approximately 25 % were synthetising DNA at any given time. An even higher proliferative activity (81% $Ki67^+$, 40% $BrdU^+$) was demonstrated in pre-B cells. In contrast, sIg^+ B cells were generally $Ki67^-$ and $BrdU^-$.

Phenotypic expression and proliferative activity of thymocytes

In the murine thymus, early observations by Weissman followed the transition from cortical to medullary thymocytes by using administration of ^3H-thymidine in vivo[27]. These observations were recently expanded by Penit who investigated the phenotype of dividing cells after in vivo administration of BrdU[28]. This study utilised a technique to perform double immunofluorescence staining with Abs to BrdU in a biotin-avidin system. $BrdU^+$ blasts were initially located in the cortex and expressed the phenotype $Lyt2^-$, $L3T4^-$ or $Lyt2^+$, $L3T4^+$. A transition from double negative to double positive cells was observed 5 days after BrdU administration. At this time labelled cells were exclusively located in the thymic medulla.

Distinct stages of differentiation are distinguishable in the thymic population in man. Most cortical thymocytes are TdT^+ and express a heterogeneous membrane phenotype. Antigens detected by McAbs of the CD1, CD2 and CD5 groups are found on the surface of these cells. Other functionally relevant structures such as CD4 and CD8 are also present on the same cells and the segregation into $CD4^+$ and $CD8^+$ cells occurs at a later stage of differentiation[29].

A small (3-8 %) subpopulation of cortical thymocytes can be identified by their size and membrane features: these are large cells, with a particularly strong

expression of nuclear TdT and prominent nucleoli and deep blue cytoplasm when stained with May Grunwald-Giemsa[30]. These large cortical thymocytes are strongly $CD7^+$, express CD5 and CD2 heterogeneously and also express HLFA antigens but no CD45 (T200) antigens[21,30,31].

The insertion into the membrane of structures which recognize antigens and transfer these stimulatory signals is a relatively late event in T cell differentiation. McAbs to the T cell receptor are now available[32]. In addition, McAbs to the closely associated CD3 molecules have been characterized. For many years, the study of the CD3 expression on thymocytes has shown a discrepancy. When used in frozen tissue sections of infant thymus, CD3 McAbs appeared to give a wide staining of both medullary and cortical thymocytes[33]. In contrast, when used in cell suspension, only a variable proportion of cells, mostly TdT^-, was stained. This discrepancy has been recently solved by several independent studies which showed that the expression of cCD3 is an early event in T cell differentiation[33-35]. Virtually all $mCD3^-$ cortical thymocytes are $cCD3^+$ when these cells are simultaneously characterized by membrane and intracellular staining using double colour immunofluorescence. Large cortical thymocytes, strongly TdT^+ and $CD7^+$ show a distinct perinuclear rim when stained with CD3 McAbs on cytospins[34,35].

We have posed the question whether cCD3 expression is also detectable in the TdT^+ cells of the BM. Double colour immunofluorescence studies in normal human BM showed that cCD3 and TdT are not found on the same cells[35]. The phenotype of the prothymocytes in man is still not known although Van Dongen and colleagues have reported the existence of a very small proportion of TdT^+ cells in the BM which express $CD7^+$ and have made the hypothesis that these cells may represent prothymocytes[36]. We could not find $CD3^+/TdT^+$ cells even in those samples which had a minute proportion (<5%) of TdT^+ cells which were weakly $CD7^+$.

Previous studies on the proliferative activity of human thymocytes have revealed a high activity within a population of large, $sCD3^-$, cortical thymocytes. We have now applied double and triple colour methods to characterize the BrdU incorporation and Ki67 expression of TdT^+, $CD1^-$ large cortical thymocytes, TdT^+, $CD1^+$ cortical thymocytes and TdT^-, $mCD3^+$ medullary cells, These studies showed that TdT^+, $CD1^-$, $sCD3^-$, $cCD3^+$ and strongly $sCD7^+$ cells represent the most actively proliferating thymocyte subset. More than 90 % of these cells are $Ki67^+$ and >70% are $BrdU^+$. The majority of TdT^+ cortical thymocytes show a lower proliferating activity, 70 % being $Ki67^+$ and 22 % $BrdU^+$. Finally, the majority of TdT^-, $sCD3^+$ cells were resting cells (Ki67 and BrdU <5%)[16,37].

The proliferative activity of malignant lymphoid cell

Similar methods were used to analyse the proliferative activity of leukaemic cells. It was noted that, as in normal BM and thymus, nuclear TdT is present during all the phases of the cell cycle, including the S phase. Secondly, the phenotype of proliferating leukaemic blasts has been characterized. CD19 and CD10 reagents together reacted with >99 % of proliferating blasts in all the null-, common- and pre B cases analysed. In T-ALL, virtually all dividing blasts regularly expressed surface CD7. Therefore in common and null ALL CD19 and CD10 reagents, and in T-ALL CD7 reagents appear to be the optimal for identifying and eliminating dividing leukaemic cells. Finally, the proportion of dividing cells in leukaemic lymphoblasts is generally smaller than it is observed in their normal counterparts (see above and ref. 16,37). This demonstrates that the principle of low leukaemic cell proliferation established in the '60s when the kinetics pattern of leukaemic versus normal myeloblasts were studied with ^{3}H-thymidine[38,39] also holds true for common and T-ALL.

The role of the microenvironment in controlling the differentiation process in the thymus and in the BM is still poorly understood. High proliferative activity and the simultaneous presence of nuclear TdT would suggest that TdT^{+} cells in the BM and cortical thymocytes are at stages of differentiation in which the generation of diversity may take place. It is unlikely that the proliferation of lymphoid progenitors is directly linked to antigen stimulation since their microenvironment lack structures which can present antigen and the immature cells lack membrane receptors for antigen. The factors which control the proliferating activity of these cells are still unknown.

It has been shown that the rearrangement process of the genes coding for Ig and T cell antigen receptor has a high rate of failure[40]. The fate of the failed B and T cells is also unknown although kinetics studies are in agreement with the thought that these cells are destined to die. The high proliferative activity together with the high content of nuclear TdT make immature B cells likely to be susceptible to spontaneous mutations which, together with a high rate of unsuccessful Ig gene rearrangement and the consequent maturation arrest could facilitate the leukaemogenic process[41]. The application of methods to characterize the proliferative status of single cells in order to study the activity of recombinant factors and stromal cells on lymphoid progenitors may provide important pieces of information to further understand leukaemogenesis.

REFERENCES

1. Lakhanpal S, Gonchoroff NJ, Katzmann JA, Handwerger BS. A flow cytofluorometric double staining technique for simultaneous determination of human mononuclear cell surface phenotype and cell cycle phase. *J Immunol Methods* 96:35, 1987.

2. Gratzner HG. Monoclonal antibody to 5-bromo-and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* 218:474, 1982.
3. Gerdes J, Lemke H, Baisch H, et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 133:1710, 1984.
4. Gerdes J, Schwarting R, Stein H. High proliferative activity of Reed Sternberg associated antigen Ki-1 positive cells in normal lymphoid tissue. *J Clin Pathol* 39:993, 1986.
5. Lokhorst HM, Boom SE, Bast BJ, Ballieux RE. Determination of the plasma cell labelling index with bromodeoxyuridine in a double fluorescence technique. *Br J Haematol* 64:271, 1986.
6. Campana D, Coustan-Smith E, Janossy G. Double and triple staining methods for studying the proliferative activity of human B and T lymphoid cells. *J Immunol Methods* 107:79, 1988.
7. Opstelten D, Osmond DG. Pre-B cells in mouse bone marrow: immunofluorencence stathmokinetic studies of the proliferation of cytoplasmic mu-chain-bearing cells in normal mice. *J Immunol* 131:2635, 1983.
8. Landreth KS, Rosse C, Clagett J. Myelogenous production and maturation of B lymphocytes in the mouse. *J Immunol* 127:2027, 1981.
9. Osmond DG. Population dynamics of bone marrow B lymphocytes. *Immunol Rev* 93:103, 1986.
10. Bofill M, Janossy G, Janossa M, et al. Human B cell development. II. Subpopulations in the human fetus. *J Immunol* 134:1531, 1985.
11. Janossy G, Bollum FJ, Bradstock KF, et al. Terminal transferase-positive human bone marrow cells exhibit the antigenic phenotype of common acute lymphoblastic leukemia. *J Immunol* 123:1525, 1979.
12. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selective antibody combinations. *Blood* 56:430, 1980.
13. Bodger MP, Janossy G, Bollum FJ, et al. The ontogeny of terminal deoxynucleotidyl transferase positive cells in the human fetus. *Blood* 61:1125, 1983.
14. Greaves M, Janossy G. Patterns of gene expression and the cellular origins of human leukaemias. Biochim Biophys *Acta* 516:193, 1978.
15. Nadler LM, Korsmeyer SJ, Anderson KC, et al. B cell origin of non-T cell acute lymphoblastic leukemia. A model for discrete stages of neoplastic and normal pre-B cell differentiation. *J Clin Invest* 74:332, 1984.
16. Campana D, Janossy G. In Mani J, Dormand J (eds). *Proceedings of the 18th International Leucocyte Culture Conference.* (In press).
17. Gathings WE, Lawton AR, Cooper MD. Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. *Eur J Immunol* 7:804, 1977.
18. Vogler LB, Crist WM, Bockman DE, et al. Pre-B-cell leukemia. A new phenotype of childhood lymphoblastic leukemia. *N Engl J Med* 298:872, 1978.
19. Campana D, Janossy G, Bofill M, et al. Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. *J Immunol* 134:1524, 1985.
20. Reinherz EL, Haynes BF, Nadler LM, Bernstein ID (eds). *Leukocyte Typing II. New York:* Springer-Verlag, 1986.
21. Campana D, Sheridan B, Tidman N, et al. Human leukocyte function-associated antigens on lympho-hemopoietic precursor cells. *Eur J Immunol* 16:537, 1986.
22. McMichael A (ed). *Leucocyte Typing III.* New York: Oxford U Pr, 1987.
23. Pezzutto A, Dörken B, Rabinovitch PS, et al. CD19 monoclonal antibody. HD37 Inhibits anti-immunoglobulin-induced B cell activation and proliferation. *J Immunol* 138:2793, 1987.

24. Clark EA, Shu G, Ledbetter JA. Role of the Bp35 cell surface polypeptide in human B-cell activation. *Proc Natl Acad Sci USA* 82:1766, 1985.

25. Pezzutto, Dörken B, Moldenhauer G, Clark EA. Amplification of human B cell activation by a monoclonal antibody to the B cell-specific antigen CD22, Bp 130/140. *J Immunol* 138:98, 1987.

26. de Gast GC, Platts-Mills TA. Functional studies on lymphocytes in adult human bone marrow. II. Isolated surface IgM-positive cells. *J Immunol* 122:285, 1979.

27. Weissman IL. Thymus cell maturation. Studies on the origin of cortisone-resistant thymic lymphocytes. *J Exp Med* 137:504, 1973.

28. Penit C. In vivo thymocyte maturation. BUdr labeling of cycling thymocytes and phenotypic analysis of their progeny support the single lineage model. *J Immunol* 137:2115, 1986.

29. Reinherz EL, Kung PC, Goldstein G, et al. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. *Proc Nall Acad Sci USA* 77:1588, 1980.

30. Bradstock KF, Janossy G, Pizzolo G, et al. Subpopulations of normal and leukemic human thymocytes: an analysis with the use of monoclonal antibodies. *JNCL* 65:33, 1980.

31. Tidman N, Janossy G, Bodger M, et al. Delineation of human thymocyte differentiation pathways utilizing double-staining techniques with monoclonal antibodies. *Clin Exp Immunol* 45:457, 1981.

32. Spits H, Borst J, Tax W, et al. Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen *J Immunol* 135:1922, 1985.

33. Link MP, Stewart SJ, Warnke RA, Levy R. Discordance between surface and cytoplasmic expression of the Leu-4 (T3) antigen in thymocytes and in blast cells from childhood T lymphoblastic malignancies *J Clin Invest* 76:248, 1985.

34. Furley AJ, Mizutani S, Weilbaecher K, et al. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. *Cell* 46:75, 1986.

35. Campana D, Thompson JS, Amlot P, et al. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. *J. Immunol* 138:648, 1987.

36. Van Dongen JJ, Hooijkaas H, Comans-Bitter M, et al. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. *J Immunol* 135:3144, 1985.

37. Campana D, Janossy G. Submitted for publication.

38. Gavosto F, Pileri A, Bachi C, Pegoraro L. Proliferation and maturation defect in acute leukemia cells. *Nature* (London) 203:92, 1964.

39. Clarkson B, Ohkita T, Ota K, Fried J. Studies of cellular proliferation in human leukemia. I. Estimation of growth rates of leukemic and normal hematopoietic cells in two adults with acute leukemia given single injections of tritiated thymidine. *J Clin Invest* 46:506, 1967.

40. Korsmeyer SJ, Arnold A, Bakhshi A, et al. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. *J Clin Invest* 71:301, 1983.

41. Greaves MF, Chan LC. Is spontaneous mutation the major 'cause' of childhood acute lymphoblastic leukaemia? *Br J Haematol* 64:1, 1986.