Leukemia Diagnosis and Testing of Complement-Fixing Antibodies for Bone Marrow Purging in Acute Lymphoid Leukemia

Dario Campana and George Janossy

In this paper a microplate method is described for diagnosing acute leukemia and for investigating the reactivity of monoclonal antibodies (MoAbs) against membrane antigens in combination with rabbit or murine antibodies to nuclear terminal transferase (TdT). The speed of this method facilitates the investigation of fresh leukemic cells from individual patients and assesses the cytolytic efficacy of the relevant MoAbs in the presence of complement (C'). Lymphoblasts (TdT +) are mixed in equal proportions with known numbers of "inert" cells, eg, RBC or nonleukemic bone marrow (BM). Following incubation with MoAbs and C' the ratio of residual TdT+ cells and inert cells is determined on cytopsin preparations. Initially, percentages of TdT+ cells are counted in a unit volume of 5,000 inert cells, followed by the scanning of >2 × 104 inert cells on entire slides. With this method more than 4 log cytoreduction of TdT+ cells is detected. The method is also applicable for studying the cytolyis of malignant B cells by using mostly monoclonal Ig expression rather than TdT for the identification of residual B cells. Ten representative patients selected from a group of >100 are reported. In some cases cytoreduction of >4 log with no identifiable residual TdT+ cells is achieved by a single C'-fixing MoAb: anti-CD10 (RFAL3) in common acute lymphoid leukemia (ALL) and anti-CD7 (RFT2) in T cell ALL (T-ALL). Other cases require cocktails of anti-CD10, anti-CD19, and anti-CD24 in common ALL or anti-CD7 and anti-CD8 in T-ALL. In T-ALL a few TdT+ cells remain that exhibit the features of normal TdT+ BM cells (CD7+, HLA-DR+). This is particularly noticeable when patients are studied in partial remission or if nonleukemic BM is used as a source of inert cells. The methods described here contribute to establishing a range of MoAbs (ie, of IgM class) and techniques for efficient purging and to comparing the efficacy of "clean-up," in remission, of common ALL, T-ALL, and B cell malignancies.

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The elimination of unwanted cells from the bone marrow (BM) during bone marrow transplantation (BMT) is referred to as "purging" or "cleansing." Monoclonal antibodies (MoAbs) able to fix complement (C') or toxin conjugates have been used successfully to remove 95% to 99.5% of T lymphocytes from allogenic BM in order to prevent graft-vs-host disease. Similar principles can also be applied for eliminating residual leukemia/lymphoma from the BM prior to autologous BMT. During this procedure proof is required to show the efficient removal of the malignant clone.

Conventional methods for investigating the efficacy of C' lysis such as dye exclusion and 51Cr release are unsuitable for detecting small numbers of residual viable malignant cells. Flow cytometry, when performed with only one single antibody, also lacks the necessary sensitivity as its lower limit of detection is 0.5% to 1%. During the last 3 years ingenious clonogenic assays have also been developed showing that MoAbs with C' or immunotoxins are capable of destroying 3 to 6 logs of cells taken from permanent cell lines of Burkitt's or acute lymphoid leukemia (ALL) origin and added as contaminants to suspensions of normal BM.

Nevertheless, these model studies do not yield information about the heterogeneity of fresh leukemic cells in terms of their susceptibility to Ab-mediated cytopathic effects.

Two aspects of purging need to be assessed when leukemic cells from patients are studied with a clinical program in mind. One is the exact proportion of leukemic cells that react with an antibody (or cocktail). The other is the efficacy of a particular cytolytic, toxic, or separating method in eliminating the population of positively labeled leukemic cells. In order to answer both of these questions, additional markers independent of the MoAb(s) used for purging have to be applied to detect leukemia. These may include (1) cytology, for the morphology of leukemic blasts such as B-ALL cells of the L3 category can be very characteristic even when seen in minute proportions; (2) detection of malignancy by chromosomal or DNA abnormality; (3) expression of membrane markers studied by additional MoAbs or by antiimmunoglobulin (anti-κ, anti-λ, or antiidiotype) Abs in B cell disorders; and finally, (4) a nuclear enzyme, terminal deoxynucleotidyl transferase (TdT) in ALL and related disorders.

In this paper we standardize methods suitable for quantitating residual ALL blast cells. While approaching this aim, three steps have been introduced: (1) A microplate method with multiple-sample handling has been adopted for rapid immunodiagnosis. (2) Murine anti-TdT MoAbs have been employed and used in double immunofluorescence (IF) combinations with MoAbs directed against membrane antigens. (3) A quantitative light microscopic test has been developed for assessing the efficacy of C' lysis in leukemia by both immunologic (TdT+) and morphological criteria. In this test leukemic cells are mixed with inert cells (eg, red cells) in known proportions. C' lysis is performed, and the loss of leukemic cells is determined following TdT staining in cytopsins. When the proportions of TdT+ (leukemic) v inert cells are counted in the control (untreated) and in post-C' samples, the depletion of leukemic (TdT+) blasts is precisely evaluated.

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MATERIALS AND METHODS

Handling of cells. Sterile samples from peripheral blood (PB) and BM were separated on a Ficoll-Hypaque gradient. Mononuclear cells were washed twice in phosphate-buffered saline (PBS) and reconstituted at 2 to 4 x 10^7/mL in PBS containing 0.2% human serum albumin and 0.2% azide (PBSA). The cells prepared for C' lysis were reconstituted at 2 x 10^7/mL in Hanks' balanced salt solution (HBSS with Ca++, Mg++, Gibco, Paisley, UK). Cytofusions were made in a Shandon cytocentrifuge (Southern Products, Astmoor, UK), fixed in cold methanol for 30 minutes, and washed in PBS. After incubation with Abs, cytofusions were covered with a 1:1 mixture of glycerol and PBS or with a medium of polyvinyl alcohol and diazobicyclooctane, which sets and retards fading.

Other slides were stained with May-Grünwald-Giemsa.

The patients' RBC were washed four times in PBS and resuspended in HBSS. Chicken RBCs (Tissue Culture Services, Slough, UK; catalog no. 101010) were used fresh or fixed in 4% formalin. The latter were washed in PBS containing 0.2% human C'. These were RFAL-3 reacting with CD10, call antigen, and RFB7 reacting with a CD20-like B cell-specific antigen. RF2T (anti-CD7) was of the IgG2 class and fixed only human C'. Additional reagents such as Abs to CD19, CD12, and CD8 were also used in the various cocktails; these were SB4, BA1, and RFT8, respectively, all of which were of the IgM class.

Heterologous antisera. Fluorochrome-labeled goat antisera to human IgM (G-anti-H-IgM–tetramethyl rhodamine isothiocyanate [TRITC], catalog no. 2020-03), x and y light chains (G-anti-H-x-TRITC, catalog no. 2060-03; and G-anti-H-y-fluorescein isothiocyanate [FITC], catalog no. 2070-02), as well as to mouse IgG and IgM (G-anti-M-IgG-FITC, catalog no. 1030-02; and G-anti-M-IgM-TRITC, catalog no. 1020-03) were purchased from Southern Biotechnology Associates, Birmingham, AL. These reagents were heavy or light chain specific, respectively. Goat antimouse IgG was eluted from an M-Ig column and conjugated with FITC or TRITC in our laboratory (G-anti-M-IgG-FITC or -TRITC) and had no activity against goat, human, rabbit, or swine Ig.

Rabbit antisera to TdT (R-anti-TdT; Supertech Inc, Bethesda, MD, catalog no. 004) reacted with HTdT in the nucleus of thymocytes and rare non-T, non-B cells of the normal human BM, but were negative (<0.1%) on circulating blood lymphocytes as well as on lymphoid cells of the human palatal tonsil. Swine antirabbit Ig (Sw-anti-R-Ig-TRITC) was from Dakopatt, Copenhagen (catalog no. R 156) and had no activity against goat, human, or mouse Ig.

The four MoAbs made against purified human TdT (HTdT-1, HTdT-2, HTdT-3, and HTdT-4, all of IgG1 subclass) were provided by Professor F.J. Bollum. The mixed cocktail of these four Abs is referred to as HTdT (total Ab content, 1 mg/mL containing approximately 0.25 mg of each Ab per milliliter). HTdT was also conjugated with biotin.

Two MoAbs used for purging were of IgM class and fixed both rabbit and human C'. These were RFAL-3 reacting with CD10, call antigen, and RFB7 reacting with a CD20-like B cell-specific antigen. RF2T (anti-CD7) was of the IgG2 class and fixed only human C'. Additional reagents such as Abs to CD19, CD12, and CD8 were also used in the various cocktails; these were SB4, BA1, and RFT8, respectively, all of which were of the IgM class.

Table 1. Diagnostic Reagents in Leukemia

<table>
<thead>
<tr>
<th>Ab (Feature)</th>
<th>Equivalent Ab</th>
<th>mol wt (Thousands)</th>
<th>Reactivity Pattern</th>
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<tbody>
<tr>
<td>Stem cell associated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. BL-3C5</td>
<td>RFDR1</td>
<td>120</td>
<td>+ + - - - - - - 20</td>
</tr>
<tr>
<td>Class II</td>
<td>RFAL3</td>
<td>100</td>
<td>+ + (-) + - - - - - - 22, 27</td>
</tr>
<tr>
<td>Myeloid</td>
<td>RFB7</td>
<td>35</td>
<td>+ - - - - - - - - 23</td>
</tr>
<tr>
<td>3. My9, MSC2</td>
<td>UCHM1</td>
<td>52</td>
<td>+ - - - - - - - - 28, 29</td>
</tr>
<tr>
<td>4. UCHM1</td>
<td>-</td>
<td>-</td>
<td>some +</td>
</tr>
<tr>
<td>Common ALL and B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. anti-CD19</td>
<td>-</td>
<td>-</td>
<td>+ - - - - - - 22, 27</td>
</tr>
<tr>
<td>6. anti-CD10</td>
<td>RFAL3</td>
<td>100</td>
<td>+ - (±) + - - - - - - 5, 22</td>
</tr>
<tr>
<td>7. anti-CD20</td>
<td>RFAL3</td>
<td>100</td>
<td>+ - - - - - - - - 22, 27</td>
</tr>
<tr>
<td>8. anti-CD20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T cell associated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. anti-CD7</td>
<td>RF1</td>
<td>65</td>
<td>+ - - - - - - - - 23</td>
</tr>
<tr>
<td>10. anti-CD2 (T11)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11. anti-CD5 (T3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12. anti-CD5 (T1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The reagents were used in a microplate assay system. On the basis of the results additional Abs are used for further analysis.

Abbreviations: AML, acute myelogenous leukemia; B-NHL, B cell non-Hodgkin's leukemia; T-CLL, T cell chronic lymphocytic leukemia.

*AMLs are heterogeneous in terms of HLA-DR expression.
†A few T-ALL cases weakly express CALL antigen.
‡Some AMLs carry p40 (CD7) antigen.
§B cells are heavy or light chain specific, respectively.
∥A few T-ALL cases are weakly membrane CD3 (T3)-positive (but many show cytoplasmic CD3).
#T-CLL is frequently CD5 (T1)-negative.

#B-CLL carries CD5 (T1) antigen.
Avidin was bought from Sigma Chemical Co, St Louis (catalog no. A-3275) and conjugated to TRITC.

Double IF assays. Leukemic blasts were labeled in suspension with MoAbs using FITC-conjugated second layers. Then cytopsins were made and fixed in cold methanol. The cells were restained with anti-TdT and TRITC-conjugated second layer. Four assays were studied: (1) MoAb + G-anti-M-Ig-FITC in combination with R-anti-TdT + Sw-anti-R-Ig-TRITC; (2) MoAb of the IgM class + G-anti-M-Ig-TRITC in combination with HTdT (IgG) + G-anti-M-IgG-TRITC; (3) MoAb + G-anti-M-Ig-FITC–labeled cells cytocentrifuged and then incubated with normal mouse serum (in order to block binding to G-anti-M-Ig) followed by biotinated HTdT Ab + avidin-TRITC; and (4) the same as 3 except that HTdT was followed by G-anti-M-Ig-TRITC (Fig 1).

Microplate method. Fifty-microliter aliquots of a 2 to 4 x 10^6/mL suspension (1 to 2 x 10^5/well) were placed into 12 microwells in a U-bottomed microplate (Sterilin, UK; catalog no. M2A4) with a repeating dispenser (2 mL total volume; Jencons, UK; catalog no. H9/255-22). Twelve (2 x 6) MoAbs (Table 1) kept in an adjacent plate as a row of concentrated stock solution and diluted with PBSA prior to use were transferred in 50-μL volumes onto the cells with a Titertek multipipette (using six of the eight available channels; Flow Labs, UK; catalog no. 77-869-00). The individual wells on the whole plate were covered with an adhesive sheet and gently shaken while incubating for ten minutes at 20 °C. The wells were topped up with 100 μL PBSA, spun in a centrifuge (Beckman TJ-6 with plate carriers) for 30 seconds at 1,500 rpm, and the supernatant was removed by inverting the plate. The cells were resuspended on a plate shaker and washed four times with cold PBSA. Cells were then incubated again for ten minutes with diluted G-anti-M-Ig-FITC (50 μL) and washed four times. After the last wash, 5 to 6 μL PBSA (with azide) was added to each of the small cell pellets using a smaller Titertek multipipette (catalog no. 77-858-00), and 2 μL of resuspended cells were transferred, as two rows of six samples, onto a polytetrafluoroethylene (PTFE)-coated multisport slide (catalog no. PH001; Hendley, Essex, UK). One slide (12 samples) for each patient was placed into formalin vapor for ten minutes, dried, and covered.

These first results identified which of the 12 MoAbs had reacted with the largest proportions of blast cells. Cells already membrane labeled were then lifted from some selected relevant wells in order to retain blasts with TdT- and TRITC-conjugated second layers.

Standardization of C. Thirty-day rabbit-C (TDRC) was obtained from 1-month-old rabbits (Research Sera Ltd, UK). In the presence of anti-T cell MoAbs of the IgM class, a cocktail of CD6 (RFT12) and CD8 (RFT8) TDRC used at 33% final dilution destroyed >98% of the T lymphocytes. This demonstrated the lytic power of the C batch. At the same time B lymphocytes or myeloid colony-forming cells were not affected, indicating the lack of nonspecific toxicity.6 Batches of TDRC were kept at 70 °C.

RESULTS

Performance of anti-TdT MoAbs. MoAbs to TdT (HTdT-1, -2, -3, and -4) and R-anti-TdT were studied on suspensions of normal BM (three samples), tonsil (five samples), thymus (three samples) and PB (five samples). All four MoAbs and R-anti-TdT showed identical reactivity: 2% to 3% positivity in BM, 59% to 77% in thymus, and no positive cells in tonsil and PB. When tested on ALL (14 cases), all four MoAbs gave positive staining: HTdT-1 showed strong, HTdT-3 and -4 moderate, and HTdT-2 weak nuclear labeling. The combination of all four (HTdT) reagents gave the strongest staining, comparable to R-anti-TdT. HTdT did not show positivity on seven cases of AML (My-9+ and TdT− when tested with R-anti-TdT).

In the next experiments R-anti-TdT was used in double combination with HTdT. In suspensions of thymocytes (two samples), normal BM (two samples), and ALL (five cases), widely different numbers of positive cells (2% to 3% in normal BM and >60% reactivity in thymus and ALL) were seen, and still the two reagents showed concordant staining on all nuclei (>1,000 cells scanned in each sample). The R-anti-TdT showed stronger cytoplasmic staining on some...
myelocytic-granulocytic cells than HTdT, but this weak extra staining did not interfere with the evaluation of results. Previous studies have already revealed the correlation between nuclear TdT staining and TdT enzyme activity measured by biochemical assay in ALL and AML\(^2\) and also showed the irrelevance of weak cytoplasmic TdT staining.

In the next experiment three combinations for membrane and nuclear labeling were tested (see Methods). In combination 2, the strong labeling with RFAL-3 (anti-CD10; IgM class + G-anti-M-Ig-M-TRITC) did not interfere with HTdT visualized by G-anti-M-IgG-FITC (heavy chain specific). In combination 3, RFAL-2 (anti-CD10, IgG2 class) and HTdT-biotin showed no membrane-nuclear cross-reactivity, and the nuclear HTdT-biotin plus avidin-FITC staining was again strong. The individual anti-TdT Abs did not

Standardization of artificial mixtures of cells. After having demonstrated the suitability of MoAbs to TdT, tests were set up to investigate the efficacy of C' lysis in leukemic samples taken at presentation. Cells (10\(^6\) cells in 50 \(\mu\)L HBSS) were placed into U-bottomed microwells, and MoAbs as culture supernatants were added in 50-\(\mu\)L volumes at saturating conditions and incubated for 15 minutes at 20 °C. The supernatant was discarded, and rabbit serum rendered the test quantitative (see the following material).

We have investigated possible errors such as selective loss or accumulation of certain cell types known to occur during cytocentrifugation. Suspensions of BM, HL-60, human RBC, and chicken RBC were mixed with ALL blasts in near equal numbers and cytocentrifuged (Table 2). All blasts appeared to be slightly diluted out (19.9% and 17.5% ALL loss) by the accumulating larger myeloid cells in the BM and by the HL-60 cells. As a contrast, ALL blasts were apparently enriched (22% enrichment) due to the relative loss of chicken RBC. This phenomenon was seen with both the fresh and formalin-fixed chicken RBC and could not be attributed to RBC lysis by heterophil antivascular antibodies present in the rabbit serum. Only minor changes (7% ALL enrichment) were, however, seen in ALL/human RBC mixtures, and for this reason we adopted human RBC inert cell populations with additional controls when normal BM was used (Table 3, patients 3b and 6b).

The results of the C' lysis assay were expressed as follows. First, the numbers of TdT+ cells were counted in an area of slides containing 5,000 inert cells (human RBC) after treatment with relevant cytolytic MoAb + C'. This value is referred to as N1. Next, the TdT+ cells were counted per 5,000 inert cells after treating an aliquot of the same suspension with an irrelevant, ineffective MoAb + C'. This value is referred to as N2. The N1/N2 ratio gives the percentage of surviving TdT+ cells. After this counting, in samples with <0.02% residual leukemia the whole slide and an additional duplicate cytopsin preparation of the same C'-treated sample were scanned and residual TdT+ cells, if any, recorded among >2 x 10^3 RBC. Thus this method is well suited for studying >4 log cyto reduction.

Additional controls were also performed. These were proportions of TdT+ cells per 5,000 inert cells incubated in the absence of MoAb or in the absence of C'. Finally, the study concluded by recording the following parameters: (1) proportions of TdT+ cells with membrane labeling (inefficiency of C', Fig 2C), (2) proportions of residual TdT+ blasts without membrane labeling (lack of Ab cover, Fig 2D), and (3) proportion of residual TdT+ blasts (in cases of mixed lymphoid/myeloid leukemia, Fig 2E).

**Purging with MoAbs and rabbit C'**. In Table 3 typical observations are shown from ten patients with ALL selected from >100 cases. Patients 1 to 4 had common ALL based on the results of microplate assay and the TdT test: BI-3C5\(^*\), HLA-DR\(^+\), CD19\(^+\), CD10\(^+\), and TdT\(^+\), but essentially

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean</th>
<th>Percentage</th>
</tr>
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<tbody>
<tr>
<td>ALL/human BM</td>
<td>76.9 ± 2.5</td>
<td>83.2 ± 8.8</td>
<td>—</td>
<td>80.1</td>
<td>−19.9</td>
</tr>
<tr>
<td>ALL/HL60</td>
<td>81.1 ± 6.7</td>
<td>91.5 ± 9.3</td>
<td>75.0 ± 9.1</td>
<td>82.5</td>
<td>−17.5</td>
</tr>
<tr>
<td>ALL/chicken RBC</td>
<td>111.1 ± 4.1</td>
<td>104.9 ± 5.1</td>
<td>105.8 ± 6.1</td>
<td>107.2</td>
<td>+7.2</td>
</tr>
<tr>
<td>ALL/chicken RBC</td>
<td>119.3 ± 6.4</td>
<td>124.4 ± 9.6</td>
<td>122.7 ± 10.0</td>
<td>122.1</td>
<td>+22.1</td>
</tr>
</tbody>
</table>

*Figures are the percentages of ALL blasts related to a standard number of inert cells (hemopoietic, HL60, human or chicken RBC) following cytocentrifugation. The ALL/inert cell value in suspension prior to spinning was 100%. Usually 1,600 inert cells were counted in each of the eight samples (± SEM).
Table 3. Elimination of Leukemic Cells with Ab + C’

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<tbody>
<tr>
<td>Diagnosis</td>
<td>cALL</td>
<td>cALL</td>
<td>cALL</td>
<td>cALL</td>
<td>null ALL</td>
<td>T-ALL</td>
<td>T-ALL</td>
<td>T-ALL</td>
<td>mixed ALL</td>
<td>B-ALL</td>
</tr>
<tr>
<td>Blasts in sample (%)</td>
<td>97</td>
<td>95</td>
<td>97</td>
<td>48</td>
<td>95</td>
<td>93</td>
<td>98</td>
<td>80</td>
<td>95</td>
<td>88</td>
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<tr>
<td>Ab reactivity</td>
<td>HLA-DR (RFDR1)</td>
<td>95</td>
<td>96</td>
<td>94</td>
<td>59</td>
<td>97</td>
<td>70±</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>34</td>
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<tr>
<td></td>
<td>CD10 (RFAL3)</td>
<td>82</td>
<td>94</td>
<td>83</td>
<td>17</td>
<td>&lt;1</td>
<td>97</td>
<td>68</td>
<td>34</td>
<td>&lt;1</td>
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<tr>
<td></td>
<td>CD7 (RFT2)</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>34</td>
<td>2</td>
<td>94</td>
<td>97</td>
<td>83</td>
<td>1</td>
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<td></td>
<td>CD2O (RFB7)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
<td>TdT (HTdT)</td>
<td>87</td>
<td>83</td>
<td>91</td>
<td>42</td>
<td>95</td>
<td>84</td>
<td>77</td>
<td>78</td>
<td>31</td>
<td>&lt;1</td>
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<tr>
<td>Combined complement lysis and TdT test</td>
<td>3a</td>
<td>3b</td>
<td>6a</td>
<td>6b</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>N1 (relevant Ab)</td>
<td>&lt;1</td>
<td>189</td>
<td>72</td>
<td>36</td>
<td>3,200</td>
<td>4,850</td>
<td>&lt;1</td>
<td>90</td>
<td>66</td>
<td>5</td>
</tr>
<tr>
<td>N2 (irrelevant Ab)</td>
<td>4,200</td>
<td>5,100</td>
<td>5,000</td>
<td>3,600</td>
<td>4,500</td>
<td>3,000</td>
<td>5,100</td>
<td>3,500</td>
<td>4,700</td>
<td>5,000</td>
</tr>
<tr>
<td>N1/N2 (%)</td>
<td>&lt;0.01*</td>
<td>3.7†</td>
<td>1.4‡</td>
<td>1.0‡</td>
<td>71</td>
<td>875</td>
<td>&lt;0.01*</td>
<td>&lt;0.02</td>
<td>1.41</td>
<td>&lt;0.02</td>
</tr>
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</table>

*When the whole slide was scanned (>10,000 RBCs) no residual TdT+ cells were seen (>4 log kill).
†The residual blasts were RFAL3- (Fig 2C) and were fully destroyed by a fresh batch of rabbit C’ (see text; <0.01 % TdT+ cells).
‡These blasts were RFAL3- (Fig 2D), and additional CD19 (SB4) was required to obtain a >4 log kill.
§CD24 (BA1) Ab was efficient in removing these blast cells.
#Residual additional TdT- myeloblasts: 7 i % (Fig 2E).

Fig 2. Quantitative assessment of ALL blast lysis with RFAL-3 (CD10, of the IgM class) and C’. The leukemia samples (>90% blasts) are mixed with equal numbers of inert human RBC. (A) RFAL-3 and C’ have eliminated all blasts; no residual TdT+ cells are seen (see also patient 1 in Table 3). (B) Irrelevant MoAbs and C’ are used, and TdT+ blasts survive. (C) The lysis is not fully effective, and a few TdT+ cells remain that carry CD10 (cALL antigen, see arrow; from patient 2). The asterisk shows a dead lysed blast. (D) The residual TdT+ cells lack RFAL-3 (CD10) reactivity (patient 3). (E) A mixed leukemia is shown. The blast cells are not lysed by RFAL-3: TdT- myeloid blasts (patient 9 in Table 3). Photographs are double (A, B) or triple (C, D) exposures of the same field with IF and phase contrast.
blasts were not clearly identifiable by morphology and masqueraded as lymphocytes. The purging was repeated with a mixture of MoAb RFAL-3 (anti-CD10) and SB4 (anti-CD19), and no identifiable TdT+ cells were left (>4 log kill). Identical results were seen when instead of human RBC normal bone marrow was the source of added inert cells (patient 3b in Table 3). These results emphasize the need for using cocktails.

A sample of PB taken from patient 4 in early relapse had a mixture of normal T lymphocytes (T3+, HLA-DR−, TdT−) and leukemia (TdT+, HLA-DR+, T3−), identified in T3/TdT and HLA-DR/TdT double-marker assays. The TdT+ blast cells were heterogeneous: only 40% of these were CD10+. Only this subset of blast cells was lysed by CD10 (N1, 3,200; N1/N2, 71%). By adding further Abs to CD19, CD9, and CD24, larger percentages of TdT+ blasts were eliminated, but a few always remained (N1/N2, >10%). We could not find a fully efficient Ab cocktail for purging the leukemia of patient 4.

Patient 5 had a CALL antigen−negative null ALL (CD10, totally negative; HLA-DR+, TdT+). Consequently, the incubation with RFAL-3 was ineffective (N1/N2, 97%). On the other hand, the blast cells were CD19+, CD24+. Good lysis (>4 log cytoreduction) was achieved in this patient with anti-CD24 (BA1 MoAb).30

Patients 6 to 8 had T-ALL: CD7+, mostly CD2+, and essentially negative with MoAbs detecting other non-T antigens (except a variable CD10 expression, see patient 6). Following the incubation with RFT2 (anti-CD7) and C’, in all three cases a drastic reduction of TdT+ cells was observed. In patient 6 the lysis of TdT+ cells was complete (>4 log). When the test was repeated (in 6b) using admixed normal BM, 2.6% residual TdT+ cells were seen, but these were all HLA-DR+, CD7− (>105 TdT+, HLA-DR− cells counted). It is therefore concluded that the specific lysis of T-ALL was >4 log in the presence of both human RBC and BM. In patient 7 the residual leukemia was 1.4% (N1/N2). These cells were CD8+, and the incubation with RFT2 (anti-CD7) + RF8T [anti-CD8]) cocktail was fully efficient (>4 log kill). In the BM sample of patient 8, five residual TdT+ cells were observed per 5,000 RBC (0.1% of TdT+ blasts, N1/N2). This BM, only partially involved, contained residual HLA-DR+, CD7−, TdT+ cells (normal BM precursors). These were, again, preserved during the treatment with MoAb to CD7 and C’.

It is relevant that in the unpurged samples of patients 2, 6, and 7 morphologically identifiable blast cells (10% to 22%) were TdT− but still expressed the relevant leukemia-associated membrane antigens: CD10 in patient 2 (see arrow in Fig 1A), CD7 in patient 6, and CD7 + CD8 in patient 7. Thus the proportions of residual TdT− blast cells after the purge were low. By contrast, the BM smears from patient 9 had a mixed lymphoid/myeloid blastic transformation of a chronic granulocytic leukemia (Philadelphia chromosome−positive). The two blast cell populations were CD10+, TdT+ lymphoid, and MCS-2+, TdT− myeloid. After having been incubated with RFAL-3 (anti-CD10) and rabbit C’, the purged sample had few TdT+ cells (N1: 66 representing 3.3% of TdT+ leukemia). Nevertheless, 71% leukemic cells, TdT− myeloid blasts, were seen (Fig 2E).

Finally, patient 10 had 88% blasts (L3 morphology, RFB7+, HLA-DR+, IgM+, TdT−). B blasts were eliminated by RFB7 and C’ as was confirmed by the disappearance of IgM+ cells (N1/N2, <0.025%).

**DISCUSSION**

MoAbs of different isotypes are available against the same leukocyte differentiation antigens (CD clusters).22,23 Their use with isotype-specific second layers together with the availability of directly labeled and biotin-conjugated Abs facilitates the clinical applications of double-color IF systems.5,33 The microplate method summarized before is useful for the following reasons: (1) This rapid assay with multiple sample-handing yields results for 12 Abs within less than 1 hour. This first round includes the CD groups of MoAbs that are also available commercially and uniformly used in many laboratories (Table 1).22,23 (2) The analysis selects relevant membrane-labeled blasts for immediate double labeling with anti-TdT in order to define the immunologic diagnosis in leukemias with low counts and with mixed features (see patients 4 and 9, respectively, Table 3). (3) Finally, following the rapid diagnosis, the C’-mediated lysis with C’-fixing MoAbs and their cocktails is used as the necessary first step to investigate in individual patients the feasibility of BM purging during autologous BMT.

In order to make these assays quantitative and sensitive, two features have been introduced. First, after the leukemic cells were mixed with known numbers of “bystanders,” or inert cells, in roughly equal proportions, their numbers were counted in a unit volume of inert cells (eg, 5,000 cells) before and after C’-mediated lysis. In cases with <0.02% residual leukemia, additional inert cells have been scanned in an even larger area of cell spreads (>2 x 106) in order to observe >4 log kill. It has been demonstrated before that the simplest inert cells are human RBC, but the C’-mediated lysis is also effective in the presence of normal BM (Table 3, patients 3b and 6b). Second, to follow leukemic cells through this process, a robust marker, independent of the Abs used for C’ lysis, was employed. Anti-TdT antibodies10 are well suited for this purpose as they detect nuclear antigens in immature lymphoid cells and their malignant counterparts. This cellular localization helps the rapid scanning of cytopsin spots on slides. We have demonstrated three different membrane marker−TdT combinations that employ MoAbs as primary reagents. Other independent membrane markers such as monoclonal Ig for malignant B cells in patient 10 or cytoplasmic staining with MoAbs to CD2234 are also suitable.

During the standardization of the purging assay two surprising observations were made. First, it has been shown that the method is suitable to identify even single TdT+ cells on slides carrying >2 x 106 inert cells. Thus the study of >4 log kill is feasible. Four examples have demonstrated how to exploit such sensitivity: (1) Patient 8 had originally presented with T-ALL, and after efficient purging using RFT2 MoAb (anti-CD7), the few residual TdT+ cells (0.1%)
expressed the phenotype of normal BM precursors. (2) In patient 2 the assay gave an early indication about the partial loss of C' activity in the stored rabbit serum, but a new batch completely cleared TdT+ blasts. (3) Our assay was necessary to search for MoAb cocktails that provided an apparently complete lysis of TdT+ leukemia in the presence of human C' (Janossy, Campana, and Bekassy; manuscript in preparation). (4) Finally, in samples from patients 3 and 7 the single MoAbs used (RFAL-3 and RFT2, respectively) were insufficient to give full lysis with rabbit C' (1.4% residual TdT+ cells in both). With the help of this assay a more efficient MoAb cocktail was found in patient 7. It can therefore be concluded that the sensitivity of the purging assay is useful.

The other unexpected observation has been that in relatively large proportions of leukemias an effective (>4 log) elimination of TdT+ cALL and T-ALL blast cells could be achieved with rabbit C' and single MoAbs (48% of ALL cases tested) or with MoAbs cocktails (77% of patients; Janossy and Campana, manuscript in preparation). These MoAbs and C' are now in use to decrease the malignant contamination in BM samples taken from patients in remission. These observations taken together with the excellent >3 log lysis in three BM samples harvested in relapse indicate that fresh leukemic cells show the same or even increased sensitivity to C'-mediated lysis when compared with cell lines.12,13

Our study is in agreement with previous observations that, partly by the help of clonogenic assays obtained with continuously growing cell lines of different (ie, cALL, T, or B) origin, have established the conditions for C'-mediated lysis. These include two rounds of C' incubation for 45 to 60 minutes each and the use of DNase.6,11,12 In addition, we used 30-day rabbit C' taken from 1-month-old animals with low levels of maternal Ig. This nontoxic C' source is easy to standardize. In all these studies the cytoreduction of MoAb-reactive cells was mostly >4 log11,12 although certain cell lines such as Ly6711 and KM312 were relatively resistant with only a 1 to 2 log decrease in spite of good MoAb reactivity. It remains unsolved whether this resistance has been acquired during the selection of cell lines or whether it represents the inherent features of some cases of leukemia/lymphoma.

Most of these clonogenic studies have not been designed to investigate the elimination of unwanted cells in a clinical setting. Only very recently has it become possible to introduce liquid culture assays for freshly established lines of Burkitt origin that still closely reflect the features of the original tumor.35 With the help of this test it has been possible to demonstrate a >6 log kill with MoAb cocktails of RFB7, SB4, and Y29/55, a cytoreduction that can be achieved with magnetic beads only when this technique is most carefully standardized (M. Favorot and I. Philip, manuscript in preparation). It is more difficult to grow ALL blasts freshly obtained from patients.36,37 The serum and growth factors are not yet fully characterized, and the plating efficiency is lower than with Burkitt's lymphoma.

In conclusion, the techniques described here will contribute not only to the selection of patients but also to that of the therapeutic MoAbs that fix rabbit and human C'. It is envisaged that our methods will help to find the tailor-made conditions for purging the BM in individual patients including the quality control of the clinical procedure, while the complementary clonogenic assays for ALL, perhaps after further development for reproducibility, may yield additional results for confirming the degree of depletion above the 4 log range of cytoreduction, as has been recently demonstrated in fresh explants obtained from Burkitt's lymphomas.35

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