Cellular Phenotypes of Normal and Leukemic Hemopoietic Cells Determined by Analysis With Selected Antibody Combinations

By G. Janossy, F. J. Bollum, K. F. Bradstock, and J. Ashley

Individual leukemic cells and the corresponding rare normal cell types in nonleukemic bone marrow were analyzed with various combinations of antisera (labeled with different fluorochromes: TRITC and FITC). Double staining for membrane la-like molecules (TRITC) and nuclear terminal transferase (FITC) was a very useful combination that distinguished common non-T, non-B ALL (la', TdT') and thymic ALL (la', TdT') from the rare cases of B ALL (la', TdT') and from AML (frequently la', TdT'; in some cases la, TdT'). Additional antisera (such as anti-ALL, anti-HuTLA, anti-immunoglobulin reagents, etc.) confirmed the diagnosis and further characterized the leukemic blasts. la', TdT' cells could be observed in low numbers in normal and nonleukemic regenerating marrow and were probably normal precursor cells; this reagent combination was, therefore, not useful for monitoring residual non-T, non-B ALL blasts in treated patients. Other marker combinations detecting pre-B ALL blasts (double staining for cytoplasmic IgM and nuclear TdT) and Thy-ALL blasts (HuTLA', TdT') were, however, virtually leukemia specific in the bone marrow and could be used to effectively monitor residual leukemic cells throughout the disease. These combined single-cell assays are not only economical and informative but are also important for assessing the heterogeneity of leukemia and for standardizing new mouse or rat monoclonal antibodies for diagnosis.

ANTI-SERA MADE AGAINST leukemia-associated antigens and lymphoid differentiation antigens of the T and B lymphoid lineages have become increasingly useful in diagnosis during recent years. A panel of reagents (Table I) has now been standardized in several laboratories, and the frequency and variability of leukemic cell types using this reagent panel have been established. Since these reagents are becoming available commercially or soon will be available as monoclonal antibodies produced by mouse (or rat) B-cell myeloma hybrids, it is appropriate to summarize current findings in this area.

These new immunologic reagents (anti-ALL serum, anti-la-like serum, anti-HuTLA serum, antiserum to terminal deoxynucleotidyl transferase [TdT], etc.) have complementary roles in the differential diagnosis of common acute lymphoblastic leukemia (cALL), thymic ALL (Thy-ALL, also referred to as T-ALL by others), and acute myeloid leukemia (AML). Diagnostic accuracy can, therefore, be increased when antisera are used in combinations labeled with different fluorochromes in immunofluorescence (IF) tests. For this reason, during the last 15 mos we have investigated the applications of these combined IF assays for clinical leukemia diagnosis. It has become apparent that the immunologic tests we use are not only economical and informative about the type of leukemia, but are also the method of choice in four further areas: (1) analysis of heterogeneity in the antigenic make-up of leukemic blasts within the malignant populations in individual patients; (2) comparison of leukemic cells with corresponding normal cell types found in normal and regenerating hemopoietic and lymphoid organs; (3) identification of abnormal cells in certain restricted tissue sites (e.g., cerebrospinal fluid, testis); and (4) rigorous follow-up of patients during apparent remission and the identification (in certain leukemic types) of residual leukemic cells present in minute proportions.

MATERIALS AND METHODS

Conventional Classification of Leukemic Cases

In untreated cases of leukemia at presentation, peripheral blood or bone marrow aspirate smears were stained with Romanovsky stains and cytochemical analysis performed with standard methods for periodic-acid Schiff, Sudan black, and/or myeloperoxidase, chloroacetate esterase, and nonspecific esterase using alpha-naphthyl acetate. Cases of undifferentiated leukemia were defined as those with undifferentiated morphology lacking definite cytochemical evidence of myeloid or lymphoid differentiation.

Preparation of Cells

Direct immunofluorescence (H') was used with anti-immunoglobulin (Ig) reagents, and other antigens were tested by indirect IF, as described previously. Briefly, bone marrow leukocytes were separated on Ficoll-Hypaque and washed; 10⁶ cells in 50μl phosphate-buffered saline (PBS; pH 7.2) were incubated with antisera diluted 1:10–1:20 or with pure antibodies used at 0.2–1 μg/μl concentration (15-min incubation at 20°C). After washing, cells were stained with...
second layer antiserum coupled to tetracythylrhodamine-isothiocyanate (TRITC; dilution 1:10—1:20). Cytocentrifuge spreads were made, fixed in cold methanol for 30 min, transferred to cold PBS for at least 30 min, and stained for nuclear TdT. After incubation with purified rabbit anti-calf TdT antibody (0.1 μg/μl; 15 min in a humidified chamber at 20°C), the smears were washed in PBS (30 min) and stained with purified goat anti-rabbit Ig antibody coupled to fluorescein-isothiocyanate (FITC; 15 min, 20°C). Alternatively, the smears of fixed cells were stained indirectly for TdT and directly for IgM (using goat antibodies to human IgM coupled to TRITC). After final staining, the slides were washed again (30 min), covered with glycerol, and sealed under a coverslip. The stained smears were examined with a standard 14 Zeiss microscope with 63 Phase objective and I/F epifluorescence condenser equipped with selective filters for FITC (green) and TRITC (red).

Unconjugated Antibodies

The panel of antibodies used in this study is listed in Table 1. Rabbit antibodies to homogeneous calf TdT (R-anti-TdT) were eluted from a TdT immunoabsorbent column as previously described. Nuclear staining is observed in ALL blast cells and lymphoid blasts during blast crisis of CML, with this reagent. Mo-1 Myeloid leukemias, lymphomas, and leukemias of B-cell type are negative. In normal tissues, cortical thymocytes and a small proportion of bone marrow cells are positive, while myeloid cells and lymphoid cells from peripheral organs are TdT negative. Rabbit antiserum to non-T, non-B ALL blast cells (R-anti-ALL) was made by injected non-T, non-B ALL blasts “coated” with antilymphocyte serum. The immune serum was absorbed with red cells, tonsil cells, adult marrow, AML, and thymocytes before use. The determinant(s) reacting with anti-ALL serum is on a single polypeptide (mol wt 95—100,000), which is shared by most cases of non-T, non-B ALL, 10% of Thy-ALL cases, and lymphoid blasts in blast crisis of CML.

Conjugated Antibodies

Goat IgG against rabbit IgG coupled to FITC (G-anti-R-FITC; fluorescein-protein ratio 3.0—3.5) was prepared as described previously. Goat IgG against rabbit IgG coupled to TRITC (G-anti-R-TRITC) and goat anti-mouse IgG coupled to TRITC (G-anti-M-TRITC) were purchased from Nordic Immunological Laboratories, Maidenhead, U.K. Goat IgG anti-human IgG coupled to FITC (Behringwerke), sheep IgG against chicken IgG coupled to FITC(S-anti-C-FITC) and to TRITC (S-anti-C-TRITC) were absorbed with pig liver powder. Goat IgG anti-human IgM was passed over IgG and IgA columns, purified by elution from an IgM-adsorbent, and then coupled to TRITC (G-anti-IgM-TRITC).

Combinations of Reagents

The most frequently used reagent combinations were as follows: (1) C-anti-la TRITC (in suspension) →TdT-FITC (in smear); (2) C-anti-la TRITC→R-anti-ALL-FITC (both in suspension); (3) R-anti-HuTLA-TRITC (in suspension) →TdT-FITC (in smears); (4) M-anti-HTA-1→FITC (in smear); (5) goat anti-Hu-Ig-TRITC (in suspension for surface immunoglobulin) →TdT-FITC (in smears); and finally, (6) G-
Fig. 1. Staining of leukemic cells for Ia-like (p28.33) membrane antigens and nuclear terminal transferase (TdT) in immunofluorescence test. Leukemic cell suspensions were stained with chicken anti-Ia antibody plus sheep anti-chicken-Ig-TRITC (red), and smeared in a cytocentrifuge. Smears were fixed and restained with rabbit anti-TdT plus goat anti-rabbit-Ig-FITC (green). (A–D) These cases contain blasts showing the phenotype of non-T, non-B ALL: Ia⁺, TdT⁻. These blast cells were HuTLA⁺, SmIg⁻. (E) Ia⁺, TdT⁺ blast cells from a case of Thy-ALL; these blasts were HuTLA⁻ (see Fig. 5). The 2 Ia⁺ cells are TdT⁻ and probably not leukemic. (F) Ia⁺, TdT⁺ blast cells from a case of AML; these blasts had Sudan black and peroxidase activity. Further details: (A) A presentation sample from a child with intermediate sized blast cells (Ia⁺, TdT⁻) uniformly expressing cALL antigens (not shown). (B) A sample from a child with lymphoid blasts heterogeneous in size, TdT, and cALL expression. The asterisks show TdT⁺, Ia⁺ blasts and arrows point to TdT⁻, Ia⁻ blasts. The labeled Ia-like membrane antigens show polar distribution (cap formation). (C) A case of undifferentiated leukemia: the Ia⁻, TdT⁻ phenotype indicates non-T, non-B ALL (although the blasts are cALL⁻). The 69-year-old male patient responded well to vincristine-prednisolone therapy. (D) Lymphoid blast crisis from a Ph₁-positive patient; only 50% of the Ia⁻ blast cells are TdT⁺ (these were cALL⁺; not shown).
anti-Hu-lgM-TRITC (in smears for cytoplasmic IgM)—TdT-FITC (also in smears). The membrane and nuclear staining could be distinguished in all combinations including No.3. Here, the membrane was stained with both G-anti-R-TRITC (strongly) and G-anti-R-FITC (weakly). This is clear from the orange (red + green) color of membrane staining in Fig. 5A,B. (We have recently started to use horse and mouse anti-HuTLA antibodies with the corresponding TRITC-labeled second layers in order to avoid the double membrane marking.)

**Specificity Controls**

Normal rabbit plus chicken sera (first layer) and G-anti-R-TRITC plus S-anti-C-TRITC (second layer) was followed by TdT staining or by staining of smears with G-anti-R-FITC alone. The staining for TdT and for CyIgM was completely blocked when smears of NALM-1 cells (a TdT⁺, CyIgM⁺ human pre-B-lymphoid cell line) were incubated with antibodies in the presence of the relevant purified antigens (TdT and IgM, respectively).²⁴

**Rosetting With Sheep Erythrocytes (E)**

Neuraminidase-treated E were mixed with leukocytes, pelleted, and incubated for 1–2 hr at 20°C. Rosettes were counted only in suspensions as described previously¹² and not in cytocentrifuge preparations, for on slides some red cells were deposited in the close vicinity of leukocytes and these “pseudorosettes” could not be confidently distinguished from the genuine E-rosettes.

**RESULTS**

**Principal Phenotypes of Leukemic Populations**

Leukemic cell populations were classified according to their dominant phenotype by staining for Ia-like membrane antigen (TRITC) in suspension followed by staining for nuclear TdT (FITC) in smears. Major population Ia⁺,TdT⁺ blast cells were observed in common non-T, non-B form of ALL (Fig. 1A and B), in a proportion of acute undifferentiated leukemia (AUL, Fig. 1C), and in lymphoid blast crisis of Ph¹-positive CML (Fig. 1D). Additional combination stains show that the Ia⁺,TdT⁺ blasts in cALL are negative with antisera to human T-lymphoid antigens (HuTLA⁻) and to membrane Ig (SmIg⁻). In marked contrast, Ia⁺,TdT⁻ lymphoid blasts constitute the malignant population in Thy-ALL (Fig. 1E); these blasts formed E-rosettes and were HuTLA⁺. Ia⁺,TdT⁻ lymphoid blasts were present in the rare cases of B-type ALL, and these cases had membrane Ig (SmIg⁺). The majority of AML and AMML cases, some AUL patients, and most cases of myeloid blast crisis were predominantly Ia⁺,TdT⁻ (Fig.1F) or, less frequently, Ia⁺,TdT⁺.

The Ia/TdT combination can be run on as few as 1–3 x 10⁶ cells if cell losses (due to adherence to plastic) during Ia staining are minimized by using small glass tubes.

**Single-Cell Analysis of Non-T, Non-B ALL Blast Cells**

A triple marker combination was used to simultaneously detect cALL antigen (membrane staining; TRITC), Ia-like antigen (membrane staining; FITC), and TdT (nuclear; FITC). In 5 of 6 childhood ALL cases >80% of leukemic cells expressed all three
markers (Fig. 2A). One of these cases had 45% leukemic cells in the sample. In one case, blasts were Ia⁺,TdT⁺, but cALL antigen negative (cALL⁻).

For routine diagnosis, it is expedient to use two simultaneous combinations: the Ia-TdT staining (as shown above) and a double staining (in suspension) for Ia antigen (TRITC) and cALL antigen (FITC).

Thirty-nine cases of non-T, non-B ALL were analyzed at presentation. In 23 cases (18 children and 5 adults, one of which was Ph¹ positive) >90% of blasts expressed all 3 markers (Ia⁺,TdT⁺,cALL⁺), although the intensity of staining showed some variation within each leukemic population with all 3 reagents. Another 5 patients (1 child and 4 adults) also showed a homogeneous population of Ia⁺,TdT⁺ blasts (>90%), but cells from these patients did not stain for cALL antigen (cALL⁻, “null” ALL ²⁵). One baby (1-yr-old) had Ia⁺,cALL⁺ blast cells that were all TdT⁺.

A striking observation was the heterogeneity of blast cells in 10 of the 39 samples studied. Four samples (from 2 children and 2 adults) contained Ia⁺,TdT⁺ lymphoid blasts with a heterogeneous expression of cALL antigen (only 15%–67% of Ia⁺,TdT⁺ blasts were cALL⁺). Three samples (from 1 child and 2 adults) contained mixtures of Ia⁺,TdT⁺,cALL⁺ and Ia⁺,TdT⁺,cALL blasts (Fig.1B). Finally, in 3 samples (1 child and 2 adults) the cALL⁻ leukemic population contained mixtures of Ia⁺,TdT⁺ and Ia⁺,TdT⁻ blast cells. These populations had lymphoblastic morphology, although the size of blast cells in some cases was variable.

Furthermore, the existence of leukemias containing mixtures of lymphoblasts (Ia⁺,TdT⁺,cALL⁺; most frequently smaller blast cells) and myeloblasts (Ia⁺,TdT⁻,cALL larger blast cells) was also confirmed by both immunologic markers and cytochemical findings in 3 cases of mixed lymphoid-myeloid blast crisis of Ph¹-positive CML and in 3 cases of Ph¹-negative acute leukemia. On phase-contrast examination, myeloblasts and early promyelocytes showed multiple nucleoli. These blasts were Ia⁺,TdT⁻. In some of these cells that were weakly Ia⁺, a few

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**Fig. 3.** In normal and regenerating bone marrow, at least three different types of Ia⁺ cells can be distinguished: (1) TdT⁺, Ia⁺, Smlg⁺ non-T, non-B cells; (2) TdT⁻, Ia⁺, Smlg⁻ cells that include larger myeloblasts (in the corner) and early forms of promyelocytes with minimal cytoplasmic granulation (not shown); and (3) TdT⁺, Ia⁺, Smlg⁻ B lymphocytes. Cells were first stained in suspension with C-anti-Ia serum plus S-anti-C-lg-TRIC (for Ia) and with G-anti-Hu-lg-FITC (for Smlg). Smeared cells were restained with R-anti-TdT plus G-anti-R-Ig-FITC (for nuclear TdT). The same area was photographed with phase-contrast and selective filters for FITC (nuclear TdT and Smlg) and TRITC (Ia).
cytoplasmic granules could also be observed under phase-contrast. On the basis of combined IF/phase-contrast analysis (e.g., size, granulation, nuclear morphology) it was easy to identify distinct subpopulations within the leukemic population and then find these cell types in conventional smears in order to establish their respective cytochemical reactivity pattern (see detailed case report in Prentice et al. ²⁶).

**Normal Bone Marrow Cells Expressing the Phenotype of Non-T, Non-B ALL**

Five bone marrow samples from nonleukemic neonates and infants (with mild anemia, eosinophilia, suspected neuroblastoma or localized B lymphoma with no marrow involvement) contained 0.3%–10% TdT⁻ cells (mean: 3.1%). Most of these cells expressed cALL antigen and Ia-like antigen (Fig. 2A) but were SmIg⁻ and also negative for HuTLA and HTA-1, an antigen specific for cortical thymocytes. These TdT⁻ cells were frequently small lymphocytes that stained weakly for the cALL antigen when compared to non-T, non-B leukemic blasts (Fig. 2B). In nonleukemic bone marrow, TdT⁺ large cells (blasts; >10μm) were also seen (~10% of TdT⁺ cells), and ~15% of small to intermediate sized TdT⁺ cells expressed cALL moderately strongly.

Similar Ia⁺,TdT⁻ cells (0.4%–8%) were seen in all of the 10 patients studied after cessation of chemotherapy. In the patients who originally had non-T, non-B ALL (5 children and 1 adult), we were unable to decide whether TdT⁻ cells in the off-treatment marrow samples included residual leukemic cells or contained only regenerating normal cells, since Ia⁺,TdT⁻ is a normal phenotype. None of these patients relapsed during the 3-mo follow-up. Similar proportions of Ia⁺,TdT⁻ cells (presumably nonleukemic) were seen in patients off treatment for TdT⁻ hemopoietic malignancy, including 3 cases of typical AML and 1 case of lymphoma.

Two cases of non-T, non-B ALL treated with allogeneic bone marrow transplantation both had detectable Ia⁺,TdT⁻ cells in the range of 0.5%–6% 3 mo after transplantation. One case relapsed 6 mo later, the other remains in full remission 9 mo after transplantation. Furthermore, 0.1%–4% Ia⁺,TdT⁻ cells were detected 2–12 mo after allogeneic transplants in 2 cases of AML and 1 case of aplastic anemia.

It is important to note that in all normal and off-treatment marrow samples, additional cells were strongly Ia⁺ but TdT⁻ (Fig. 3). These cells were particularly heterogeneous in size. Many large Ia⁺ blasts showed the phase-contrast appearance of myeloblasts or early promyelocytes with multiple nucleoli and some cytoplasmic granularity. Other cells were small lymphocytes that proved to be SmIg⁺,Ia⁺,TdT⁻ B cells in the triple marker combination (shown in Fig. 3). Thus, the prevailing impression in normal and regenerating bone marrow samples was the coexistence of three Ia⁺ populations: (1) the Ia⁺,TdT⁺,cALL⁺ mostly small and intermediate non-T, non-B cell type; (2) the Ia⁺,TdT⁻ larger myeloid elements; and (3) the Ia⁺,TdT⁺, SmIg⁻ small B lymphocytes.

Eight relapsed leukemic cases studied contained 7.5%–95% Ia⁺,TdT⁺ cells. Five bone marrow samples had large proportions of blasts (20%, 30%, 40%, 60%, and 95%; Ia⁺,TdT⁺,cALL⁻ intermediate sized blasts or, in one case, very strongly Ia⁺⁺,TdT⁺, cALL⁻ large blasts). These findings were similar to those previously seen at the presentation of these patients and confirmed the hematologic diagnosis of the relapsing leukemia. In three cases, the proportions of Ia⁺,TdT⁺ cells were 7.5%–10% 1–2 mo before florid relapse. Interestingly, many of these were small cells of lymphocyte morphology expressing the cALL antigen with variable intensity and containing an additional heterogeneous population of Ia⁺,TdT⁻ cells.

Thus, in regenerating bone marrow and in the early stages of relapse, the phenotypes of the normal Ia⁺,TdT⁺,cALL⁻ cell types and their malignant counterparts are similar. At the present time it is impossible to make a confident assessment about the recurrence of common non-T, non-B ALL when Ia⁺,TdT⁺ cells are present at 1%–10%.

**The Pre-B ALL Phenotype**

About 25%–30% of non-T, non-B ALL cases (Ia⁺,TdT⁻, frequently cALL⁻, always SmIg⁺) express cytoplasmic IgM (pre-B ALL). These can be diagnosed by a combined staining for nuclear TdT (FITC) and cytoplasmic IgM (CyIgM; TRITC: Fig. 4A). Three of the five pre-B ALL studied showed uniform simultaneous expression of both markers in >80% of blasts (TdT⁻,CyIgM⁺). One case had a mixture of 60% TdT⁻,CyIgM⁺ and 40% TdT⁺,CyIgM⁻ blasts. The fifth case also had a mixture of 30% TdT⁻,CyIgM⁻, 25% TdT⁺,CyIgM⁻, and 45% TdT⁻,CyIgM⁻ blast cells.

**Pre-B-Cells in Normal and Regenerating Bone Marrow**

Double labeling techniques were used to investigate the expression of TdT and CyIgM in normal and
Fig. 4. Staining of pre-B ALL blast cells (A) and normal bone marrow cells (B) in smears for cytoplasmic IgM (TRITC; red) and nuclear TdT (FITC; green). Leukemic blasts of pre-B ALL type are CyIgM+, and most of the blasts are also TdT+ (A). In contrast, in the normal bone marrow (B), stained cells are either CyIgM+, TdT- (non-T, non-B cells) or CyIgM-, TdT+ (typical large pre-B-cell on the left). The CyIgM+, TdT+ cell in the lower middle area of the photograph represents a very rare cell in normal marrow. Only about 1% of the non-T, non-B and pre-B-cell population stain for both TdT and CyIgM. In right corner are 2 small B lymphocytes.

Fig. 5. Staining of Thy-ALL blast cells (A), normal thymocytes (B), and normal bone marrow (C) for HuTLA (TRITC; red) and TdT (FITC; green). Cells were stained with R-anti-HuTLA plus G-anti-R-Ig-TRITC in suspension, smeared, fixed, and restained for R-anti-TdT plus G-anti-R-Ig-FITC. Thy-ALL blasts (A) and cortical thymocytes (B) are HuTLA+, TdT+. In this thymus sample, the labeled membrane HuTLA shows polar redistribution (cap formation). In the marrow (C), only few cells stain with this reagent combination. These cells are either HuTLA-, TdT+ (non-T, non-B) or HuTLA+, TdT+ (T lymphocytes), but no HuTLA-, TdT+ doubles can be seen.
Fig. 6. Staining of Thy-ALL blast cells (A), normal thymocytes (B), and normal bone marrow (C) for HTA-1, a cortical thymocyte-specific antigen (TRITC; red) and TdT (FITC; green). Cells were stained with NA1/34 (a monoclonal mouse antibody to HTA-1) plus G-anti-mouse-lg-TRITC in suspension and restained in smears for TdT (FITC). Some of the Thy-ALL blasts express variable but frequently low amounts of HTA-1 (A), which is more abundant on cortical thymocytes (B). TdT+ cells in the marrow are HTA-1 (C).

Further tests (E-rosetting with sheep erythrocytes and staining with anti-HuTLA serum) can confirm the diagnosis. The two combinations most useful in this study were staining for membrane HuTLA (TRITC) and nuclear TdT (FITC; Fig. 5) and staining for membrane HTA-1 (a cortical thymocyte-specific antigen; TRITC) and TdT (FITC; Fig. 6). In the 8 Thy-ALL cases studied, a uniform and strong expression of HuTLA and TdT was observed on >90% blast cells (Fig. 5A). The composite phenotype of these blast cells was therefore HuTLA+, TdT+, Ia-. One case had a mixture of HuTLA+, TdT+, Ia- and HuTLA+, TdT-, Ia- blasts. None of the 8 Thy-ALL and 39 common ALL cases studied had mixtures of Thy-ALL blast cells (HuTLA+, TdT-, Ia-) and common ALL blast cells (HuTLA-, TdT+, Ia+). Thus, mixed leukemias with Thy-ALL and common ALL blast cells seem to be very rare, if they exist at all. (In the rare cases of Thy-ALL that weakly react with the rabbit anti-ALL serum the phenotype of blasts is HuTLA+, TdT+, Ia-. Again, no HuTLA-, TdT+, Ia+ leukemic blasts are present in these cases.)

The Thymic ALL (Thy-ALL) Phenotype

The Ia+, TdT+ staining of lymphoblasts at presentation (Fig. 1E) indicates that the patient has Thy-ALL. A small subpopulation (1.8%) of cells within the TdT+ population (representing only 0.01%-0.04% of all marrow cells studied) showed weak CylgM staining (Fig. 4B), frequently restricted to the perinuclear area of the cytoplasm. The vast majority of TdT+ cells (>98%) were CylgM- and most pre-B cells were TdT- (Fig. 4B). The scarcity of TdT+ pre-B-cells has been confirmed in 5 marrow samples taken from patients off treatment for non-T, non-B ALL with only about 1% of TdT+ cells expressing CylgM in all cases studied. Thus, the TdT/CylgM staining combination appears to be useful for monitoring leukemic blast cells during treatment in patients presenting with TdT+, CylgM+ pre-B ALL. More than 10%-15% TdT+, CylgM+ blasts within the TdT+ population may suggest residual leukemia or relapse of the TdT+, CylgM+ leukemic clone.
Thy-ALL blasts were more heterogeneous when studied with E-rosetting (E) and with the HTA-1/TdT combination. Two of the 9 Thy-ALL cases studied failed to form E-rosettes (E'), and in 3 additional cases, only 50%–55% of blasts formed E-rosettes. Three of the 8 Thy-ALL cases tested failed to express detectable HTA-1 antigen; HTA-1' blasts constituted only a proportion (0.5%, 4%, 16%, 32%, and 85%) of TdT' blasts in the remaining 5 cases (Fig. 6A).

**Cells With Thy-ALL Characteristics Are Not Present in Normal and Regenerating Bone Marrow**

Double staining with HuTLA/TdT and HTA-1/TdT combinations is uniquely characteristic of normal cortical thymocytes (HuTLA', HTA-1', TdT'); Figs. 5B and 6B). Medullary thymocytes and peripheral T lymphocytes are HuTLA' but HTA-1', TdT' (further details see Janossy et al.27). We have therefore analyzed normal marrow for the presence of cells expressing cortical thymocyte phenotype.

Three bone marrow samples from normal infants and adolescents and 3 samples of regenerating marrow (taken from patients off treatment for non-T, non-B ALL) were stained with HuTLA/TdT combination (Fig. 5C). No HuTLA', TdT' double-labeled cells were seen among the >1000 TdT' cells analyzed. One-half to three percent of marrow cells were HuTLA', TdT' (non-T, non-B cells) and 2%-6% were HuTLA', TdT' (marrow T lymphocytes). Analysis of the HTA-1/TdT combination confirmed that cells of cortical thymocyte phenotype were not detectable in the marrow (among >500 TdT' cells studied). TdT' cells were also HTA-1'.

**Monitoring of Patients in Thy-ALL**

The presence of cells expressing HuTLA', TdT' phenotype or HTA-1 antigen in the bone marrow is indicative of residual or relapsing Thy-ALL cells. These combinations were therefore used to monitor six patients with Thy-ALL. The salient points from this analysis are, first, that the HuTLA/TdT combination represents a sensitive test for monitoring patients with Thy-ALL. Small proportions (0.5%) of HuTLA', TdT' blasts indicated the persistence of the leukemic clone during treatment when one of the patients was considered to be in full hematologic remission. Second, the test is discriminative. In the same sample (in addition to the 0.5% HuTLA', TdT' blasts), HuTLA', TdT' cells and HuTLA', TdT' cells were also seen. These cells were most probably normal bone marrow precursors and T lymphocytes (respectively) not primarily involved in leukemia since the patient relapsed later with HuTLA', TdT' blast cells. Third, the test was economical. It has been performed on a small cerebrospinal fluid sample and established the presence of the HuTLA', TdT' leukemic clone in the central nervous system.

**DISCUSSION**

Leukemic blast cells and normal or regenerating bone marrow samples were simultaneously labeled for nuclear TdT and for various membrane antigens, most frequently human Ia-like (p28, 33) determinants. This technique provides new insights in leukemia diagnosis. The three main conclusions from our studies are as follows.

First, the analysis of individual leukemic cells with this technology gives more pertinent details than the immunologic techniques using complement-mediated cytotoxicity or membrane labeling with single color IF in cell suspension. Microscopic observations exploiting two independent immunofluorescence (IF) markers and phase-contrast appearance of smeared cells can be easily related to the “classical” morphology of leukemic cells observed in smears stained with May-Grünwald-Giemsa and special cytochemical stains. The information value of the methods used here is as great as the hematologic analysis (with May-Grünwald-Giemsa) of prelabeled cells after having been sorted on the Fluorescence Activated Cell Sorter,28-29 a technique that requires larger numbers of cells and expensive instruments.

Second, the marker combinations used readily distinguish the major types of acute leukemias (i.e., non-T, non-B ALL, Thy-ALL, B-ALL, and myeloid leukemias) and various subgroups within these major categories (e.g., pre-B ALL within the non-T, non-B group; see Table 2). Previous studies have indicated that the choice of treatment and the prognosis in these diseases is different.1,25 Furthermore, our studies clearly indicate that while some leukemias (i.e., most cases of childhood non-T, non-B ALL) show fairly homogeneous membrane marker, enzymatic and morphological characteristics, other cases can be heterogeneous. Since relapse frequently develops from a relatively drug-resistant subclone that may show phenotypic characteristics different from the drug-sensitive leukemic cells,10,31 further studies are essential to analyze whether the heterogeneity of leukemic blasts represent an important poor-risk prognostic factor. In addition, the apparent heterogeneity of membrane marker expression by leukemic blast cells may have serious implications for the use of antibodies.
against these membrane moieties in attempted removal of residual leukemic cells prior to autologous bone marrow transplantation. Clearly in a proportion of patients, mixtures of antibodies reacting with different membrane markers will be required for this purpose.

It is also interesting that in some leukemias the blast cells show rather uniform morphology, while heterogeneity can only be observed by membrane and enzyme markers; in other cases, the leukemic populations constitute mixtures of blasts that differ in both their morphological and marker characteristics. In one case of childhood ALL, mixtures of small non-T, non-B blasts (TdT\(^-\), cytoplasmic IgM\(^-\)) and typical pre-B-cells (TdT\(^+\), clygM\(^+\)) could be demonstrated with intermediate TdT\(^+\), clygM\(^+\) forms, suggesting that the non-T, non-B ALL are undergoing a partial differentiation into early forms of pre-B-lymphoid cells. Our studies also confirm previous observations that suggested that mixed lymphoid and myeloid blast cells are found in some acute leukemias\(^31\) as well as during the acute phase of Ph\(^1\)-positive CML.\(^{29,33}\) In a recent case report,\(^{17}\) the various subpopulations (i.e., lymphoid Ia\(^+\), TdT\(^+\) and myeloid Ia\(^+\), TdT\(^-\)) were further documented and showed markedly different sensitivity to different therapeutic regimes. Our detailed observations on the heterogeneity of blast cells in AML will be published separately.\(^{24}\)

The third main conclusion is that the use of antisera combinations is a particularly powerful technique for the comparative analysis of leukemic cells and their normal counterparts.\(^{4,18}\) In principle, one label (in this paper, TdT staining) is used to mark the population of interest (e.g., most blasts in ALL, or a TdT\(^+\) minority population in normal and regenerating marrow, or cortical thymocytes in normal thymus). The reactivity of marked cells with an antibody to membrane antigens can then be systematically analyzed in a series of tests using different antibodies. The phenotypes of the TdT\(^+\) populations are thus established. Our studies clearly show that both non-T, non-B ALL blasts and a subset of non-T, non-B cells in normal infant marrow express the TdT\(^+\), Ia\(^+\), ALL\(^+\), HuTLA\(^+\), HTA-1\(^+\), SmIg\(^-\) phenotype.\(^4\) Minor differences between the leukemic and corresponding normal cells can be observed, however: in many cases of non-T, non-B ALL, the majority of TdT\(^+\) cells are blasts that express ALL antigen fairly strongly. In contrast, normal TdT\(^-\) infant bone marrow cells are smaller cells of lymphocyte morphology that express ALL antigen weakly. These distinctions between the malignant and normal cell populations are nevertheless not absolute, and many TdT\(^+\) cells in regenerating marrow are blasts (~10%) and a similar proportion express ALL antigen moderately strongly. This prevents a confident immunologic diagnosis of early relapse in non-T, non-B ALL with the markers used. Our observations are in line with similar studies based on the analysis of cells reacting with anti-ALL antiserum in normal infant and regenerating bone marrow samples.\(^9,35\) It is important to note, however, that the same double-marker principle of analyzing TdT\(^+\) cells in leukemic and normal bone marrow can be used to select newly produced antibodies, e.g., synthesized by mouse/rat myeloma-B-lymphocyte hybrids (monoclonal antibodies). Some of these antibodies might eventually distinguish between leukemic and normal TdT\(^+\) cell populations of the bone marrow, but this point has to be analyzed very critically, perhaps by single-cell assays as described in this article. In fact, a recently described monoclonal anti-non-T, non-B ALL antibody seems to be reactive with non-T, non-B ALL cells only, while apparently failing to react with cells in normal and regenerating bone marrow.\(^{11}\)

While discrimination between normal and leukemic cells in non-T, non-B ALL is still not possible, our study clearly shows that in pre-B ALL (TdT\(^+\), clygM\(^+\)) and Thy-ALL (TdT\(^+\), HuTLA\(^+\), Ia\(^+\)), the existing double-marker technology is capable of identifying residual or relapsing leukemic blasts, even when present in very low numbers. In cases of pre-B ALL, the corresponding normal TdT\(^+\), clygM\(^+\) cells are exceedingly rare (Fig. 4). In Thy-ALL, the cell of origin seems to be a HuTLA\(^+\), TdT\(^-\) early thymocyte that is essentially "alien" to the normal bone marrow\(^{18}\)

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**Table 2. The Main Phenotypes in Acute Leukemia**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Typical phenotype</th>
<th>Subgroup</th>
<th>Typical phenotype</th>
<th>Subgroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Common (non-T, non-B ALL)</td>
<td>Ia(^+), TdT(^+), cALL(^+), E(^+), HuTLA(^+), SmIg(^-)</td>
<td>E cases*</td>
<td>TdT(^+), cALL(^+), E(^+), HuTLA(^+), SmIg(^-)</td>
<td>E cases*</td>
</tr>
<tr>
<td>II. Thymic ALL</td>
<td>Ia(^+), TdT(^+), cALL(^+), E(^+), HuTLA(^+), SmIg(^-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III. B-ALL</td>
<td>Ia(^+) or Ia(^+), TdT(^+), cALL(^+), E(^+), HuTLA(^+), SmIg(^-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV. AML</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*For further details see refs. 2, 17, and 18.

\(^*\)A subset of blasts reacts with HTA-1, a thymocyte-specific antigen.\(^8\)
(Fig. 5) but seems to “naturalize” to the bone marrow in Thy-ALL. Our findings also demonstrate that immunologic monitoring of certain leukemias at the level of single cells is feasible. The analytical procedure may help to assess whether or not bone marrow taken during apparent hematologic remission (e.g., in patients treated for Thy-ALL) contains residual leukemic cells. These tests will also be useful for monitoring the efficacy of the removal of these residual leukemic populations from stored marrow samples prior to reinfection into the heavily treated patients (during autologous bone marrow transplantation).

Finally, the single-cell assays described in this article detect isolated malignant cells in sites other than bone marrow, such as the cerebrospinal fluid, testis, or other tissues. These studies will be published elsewhere.19,20

ACKNOWLEDGMENT

We thank Prof. A.V. Hoffbrand, Dr. G. Prentice (Department of Haematology, Royal Free Hospital), Dr. M. Willoughby and his colleagues (Royal Hospital for Sick Children, Glasgow, Scotland) for their encouragement and collaboration; Drs. A. McMichael and C. Milstein for the gift of NA1/34 antibody; Sylvia Granger and N. Tidman of Royal Free Hospital, for preparation of fluorochromecoupled purified antibodies; and Vanessa Lipton for secretarial help.

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Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations

G Janossy, FJ Bollum, KF Bradstock and J Ashley