Analysis of Leukocyte Differentiation Antigens in Blood and Bone Marrow From Preleukemia (Refractory Anemia) Patients Using Monoclonal Antibodies

By Peter Hokland, Gitte Kerndrup, James D. Griffin, and Jørgen Ellegaard

Peripheral blood and bone marrow mononuclear cells from patients with refractory anemia (RA) or RA with sideroblasts (defined according to the revised French-American-British classification with less than 5% blast cells in the bone marrow) were analyzed using a panel of monoclonal antibodies directed against leukocyte antigens on B lymphocytes, T lymphocytes, monocytes, and myeloid cells. In the peripheral blood an increased proportion of T lymphocytes (and correspondingly a decreased proportion of B cells) could be demonstrated. However, when expressed in terms of absolute numbers, the T cell component was depressed because of severely decreased numbers of T4+ helper cells. In contrast, the absolute numbers of T8+ suppressor cells were either normal or increased in the majority of the patients. This resulted in markedly decreased ratios of T4+/T8+ cells, which were closely correlated to the number of transfusions given to the patients because of their refractory anemia. Finally, nearly all of the patients exhibited decreased numbers of cells reactive with the N901 natural killer (NK) antibody, thus explaining our earlier finding of decreased NK activity in these patients. In the bone marrow increased proportions of myeloid cells reactive with monoclonal antibodies present on immature myeloid cells (My7 and My9) were found, suggesting the presence of malignant clones. Indeed, when the numbers of My7+ cells and the morphologic evaluations of bone marrow smears at the time of diagnosis were compared to the progression of the disease, a group of patients with high numbers of My7+ cells and normal morphology could be identified that had a high probability of progression to refractory anemia with an excess of blasts or to overt acute myeloid leukemia. Thus, the use of antibodies defining leukocyte differentiation antigens might be of significant value in the diagnosis and prognostication of the myelodysplastic syndromes. These findings are discussed in relation to the pathogenesis of this potentially premalignant condition with special emphasis on possible defects in the immunologic defense mechanisms against early neoplasias.

MATERIALS AND METHODS

Patients. Thirty-two patients, 17 females and 15 males, were found to fulfill the diagnostic criteria of RA discussed in detail elsewhere; ten patients presented with ring sideroblasts (RA-S). Fifty-five percent had clonal karyotypic abnormalities when examined by chromosome-banding techniques. It should be stressed that all had less than 5% blast cells in the bone marrow and none had myeloblasts in the peripheral blood at the time of diagnosis.

Preparations of mononuclear cell suspension from peripheral blood and bone marrow. Freshly drawn heparinized venous blood was layered onto Isopaque-Ficoll (Nygaard, Oslo), and red cells and mature myeloid cells were removed by density centrifugation. The interface cells were washed twice in Hanks' buffered salt solution supplemented with 2.5% pooled human AB serum and adjusted to a concentration of 1 x 10^6/mL. Bone marrow was suspended in preservative-free heparin and diluted approximately 1:4 in HBSS. After Isopaque-Ficoll centrifugation the cells were treated as previously described for peripheral blood cells.

Monoclonal antibodies. The MoAb used in this study are shown in Table 1 together with a summary of their reactivities and original references. They were selected to give as complete a picture of the differentiation antigens on T cells, B cells, monocytes, and myeloid cells as possible. B1 (provided by Dr L.M. Nadler) is expressed on B cells and some malignant B cells including 50% of acute lymphoblastic leukemia and 40% of lymphoblast crisis cells of chronic myeloid leukemia. For the definition of the T3 pan/T cell antigen, the UCH-T1 antibody produced by Dr P.C. Beverley was used. The T4 helper/inducer antigen and the T8 cytotoxic/suppressor antigen were defined by the B66.6 and the B99.1 antibodies produced by Dr
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Antibody. For the evaluation of the myeloid component we used MoAbs of the My-series produced and characterized by J.D. Griffin. The My4 antigen is expressed on mature and immature monocytes as well as on acute monocytic leukemia cells; M7 is expressed preferentially on immature myeloid cells not including the CFU-C. My8 is expressed on more mature myeloid cells and to a certain degree on peripheral blood monocytes. The My9 antigen is expressed on immature myeloid cells including some CFU-C and BFU-E.

In contrast, the NAT-9 antibody, which exclusively reacts with mature myeloid cells, was provided by Dr. R.B. Andreasen. Finally, HLA-DR antigens were detected using the AAS-1 antibody produced in our laboratory, which reacts with a HLA-DR framework antigen.

All antibodies were used as ascites in dilutions varying from 1:50 to 1:450.

Indirect immunofluorescence assay. Blood and bone marrow cells were adjusted to a concentration of $1 \times 10^6$/mL, and 0.5 to 1 $\times 10^6$ cells were incubated for 30 minutes at 4°C with appropriately diluted ascites containing the aforementioned antibodies. After two washes in HBSS plus 2.5% AB serum, the cells were incubated with a rabbit antiserum (Dakopatts, Copenhagen) at a dilution of 1:20 at 20°C for 30 minutes. After two additional washes in HBSS plus 2.5% AB serum supplemented with 1% formaldehyde, the number of positive cells reactive with the different antibodies was scored by fluorescence microscopy after counting at least 200 cells.

RESULTS

Analysis of peripheral blood mononuclear cells. Many of the patients exhibited varying degrees of lymphopenia, resulting in decreased absolute numbers of most of the subsets measured. The group of patients was very heterogeneous with respect to B1 antigen expression since only nine of the patients were within normal range, eight patients exhibiting significantly higher numbers and 12 patients exhibiting lower numbers of B lymphocytes. In 23 of 29 patients the absolute numbers of B1+ cells were decreased. Using the anti-My4 antibody–defining monocytes, the same pattern as that seen for B1 was observed. However, very high numbers were seen in two patients who later progressed to chronic myelomonocytic leukemia (CMML) (data not shown).

In Fig 1 the results from the analysis of T cells and their subsets are given. Using the UCHT-1 antibody as a pan-T cell marker, the absolute numbers of cells were decreased in 24 out of 29 patients. With regard to T cell subsets, a marked heterogeneity was observed with respect to the expression of the T4 antigen, which is a reliable marker for the helper/inducer subset. However, strikingly decreased amounts of circulating T4 cells were seen in 27 out of 29 patients. In contrast, 14 of 29 patients showed increased numbers of cytotoxic/suppressor cells as evaluated by MoAb B.99.1 directed against the T8 antigen (Fig 1), with only seven exhibiting slightly decreased circulating T8 cells. These changes are reflected in the ratios between helper and suppressor cells (T4/T8 ratios), which were decreased in 17 of the 29 patients (data not shown). Moreover, four of the patients with normal or increased ratios subsequently converted during the observation period.

Many reasons for this disturbance in T cell subsets can be envisaged, but in Fig 2 we have related the number of transfusions given to the individual patients to their T4/T8 ratio. It will be seen that with an increasing number of transfusions a steep fall in the T4/T8 ratio is seen during the first 20 transfusions, followed by a more gradual one up to 200 transfusions. Finally, with respect to the analysis of peripheral blood, we looked at the percentage of N901-bearing cells, which have been shown to contain the NK cell fraction. A marked decrease was observed in 26 out of 29 patients (Fig 1), with extremely low numbers (less than 10% of that seen in normal donors) in 14 patients.

Since the aforementioned observed changes in the proportion of lymphocyte subsets could be of major importance in the etiology and pathogenesis of the anemia seen in these patients, we found it of major importance to evaluate the consistency of the observed abnormalities with respect to...
Apart from minor fluctuations, the aforementioned observed same patient on three different occasions, it will be seen that longitudinal measurements on peripheral blood cells from the short-term reproducibility. From Table 2, which shows longitudinal marrow.  

**Table 2. Longitudinal Determinations of Leukocyte Differentiation Antigens in RA**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Date</th>
<th>MY4</th>
<th>MY7</th>
<th>MY9</th>
<th>B1</th>
<th>T3</th>
<th>T4</th>
<th>T8</th>
<th>N901</th>
</tr>
</thead>
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<td>ER</td>
<td>PB</td>
<td>1/25/83</td>
<td>20*</td>
<td>NT</td>
<td>NT</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ER</td>
<td>PB</td>
<td>3/21/83</td>
<td>20</td>
<td>NT</td>
<td>NT</td>
<td>11</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ER</td>
<td>PB</td>
<td>5/26/83</td>
<td>17</td>
<td>NT</td>
<td>NT</td>
<td>9</td>
<td>2</td>
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<tr>
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<td>BM</td>
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<td>6</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>EB</td>
<td>BM</td>
<td>5/9/83</td>
<td>7</td>
<td>13</td>
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<td>10</td>
<td>NT</td>
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</tr>
</tbody>
</table>

Abbreviations: PB, peripheral blood; NT, not tested; BM, bone marrow.  

*Percentage of positive cells.

short-term reproducibility. From Table 2, which shows longitudinal measurements on peripheral blood cells from the same patient on three different occasions, it will be seen that apart from minor fluctuations, the aforementioned observed tendencies were very stable.

**Analysis of mononuclear bone marrow cells.** Bone marrow aspirations or Jamshidi bone marrow biopsies from the iliac crest were attempted in all patients, and suitable material was obtained in 30 out of 32 patients. In one patient, three different aspirations were performed, all of them contaminated with peripheral blood cells to such an extent that proper analysis could not be performed. In another patient, ordinary aspirations from the sternum resulted in a dry tap, and attempts to recover cells from several Jamshidi biopsies failed. The analysis of the remaining 30 patients is summarized in Figs 3 to 5; not shown are data demonstrating that the mononuclear bone marrow suspensions contained low numbers of both T and B cells as evaluated by B1 and T3 (thus excluding a gross admixture of peripheral blood in these aspirates). Cells of the myeloid lineage were evaluated using five different MoAbs, all related to different stages of myeloid differentiation. In normal humans My7 is usually expressed on less than 10% of mononuclear bone marrow cells, but it will be seen from Fig 3 that the My7 was expressed on an increased percentage of cells in 26 out of 29 patients. In contrast, the expression of My9, which is somewhat more broadly reactive, was increased in only 11 out of 29 patients. The My8 and NAT-9 antibodies react with major fractions of normal bone marrow cells, and from the data shown in Fig 4 it will be seen that between 20% and 70% of the cells from the preleukemia patients expressed these antigens, and only in a minority of cases in increased numbers. Finally, the percentages of HLA-DR-bearing cells in the bone marrows are also shown in Fig 4, and it is clear that few significant increases were observed.

To further elucidate the significance of the increased percentages of My7+ cells in the bone marrow (Fig 3), we compared them to the morphology and clinical course. Choosing the combined percentages of myeloblasts and promyelocytes as indicators of bone marrow maturity, it could be demonstrated (Fig 5) that patients with subsequent progression from RA or RA-S to RA with an excess of blasts (RAEB) exhibited high numbers of My7+ cells but usually no or modest abnormalities in morphology ($P < .01$, Wilcoxon's rank sum test). Moreover, patients with very high numbers of My7+ cells were characterized by large transfusion needs and fast disease progression either to RAEB, AML, CMML, or death resulting from severe thrombocytopenia related to bone marrow depression.

As was the case with blood specimens, short-term longitudinal studies were also performed on bone marrow cells. From Table 2 it will be seen that in a patient with slightly increased numbers of My7+ cells but stable transfusion needs the antigen expression remained stable for a 12-month period.

**DISCUSSION**

Until recently, the diagnostic criteria for myelodysplastic syndromes were ill defined, and consequently several groups of patients, including those with CMML, smouldering AML, or RAEB were grouped together in preleukemia patient studies. With the recent definition of diagnostic criteria, a group of preleukemia patients with RA or RA-S are defined as those having less than 5% blasts in the bone marrow and...
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less than 1% in peripheral blood, and in this study these diagnostic prerequisites were followed. Consequently, several patients with more than 5% blasts have been excluded from this investigation, but will be described separately.

Until now, laboratory studies in preleukemia patients have mainly consisted of cytogenetic analysis and determination of growth in semisolid agar.2-3 These studies have shown that the majority of the patients have distinct clonal abnormalities accompanied by altered growth capacities in CFU-C assays, indicating that at least some of the patients malignant clones are present. In contrast, few studies have dealt with detailed immunologic investigations in these patients in spite of the fact that overt AML develops in some of the patients at a later stage. This condition could thus provide a rare opportunity for studying the immune system at a potentially premalignant stage, and we therefore decided to undertake a detailed study of various aspects of the immunologic response in these patients. Here we have described the results from the analysis of blood and bone marrow mononuclear cells using a panel of MoAb suited for evaluating the major lineages of hematopoiesis.

Even though most malignant blood diseases originate in the bone marrow, evaluation of leukocyte subsets in the peripheral blood can yield valuable information concerning their possible progression and give indications on functional assays that could elucidate their pathogenesis. The most interesting finding emerged from the blood suppressor cells (Fig 1) that resulted from decreased numbers of T4 cells and increased numbers of T8 cells. Similar findings were recently published by Anderson et al in a smaller group of patients,16 but we have extended these findings considerably by demonstrating that such patients exhibited a marked heterogeneity, with a range of T4/T8 ratio from 0.3 to 5.2. Since suppressor cells are thought to influence erythropoiesis, this heterogeneity might be of importance in determining the cause of the anemic stage in these patients, but we have at present no data suggesting that in patients presenting with low T4/T8 ratios more severe anemia develops than in those with normal ratios. Based on these findings, studies are, however, under way to analyze the influence of T4 and T8 cells (using either fluorescence-activated sorted cells or in vitro propagated T cell clones) on autologous CFU-C and CFU-E.

The anemic stage does in some patients require frequent blood transfusions, and we have—in light of the recent findings of altered T4/T8 ratios in hemophilic patients given factor VIII concentrate15—tried to correlate the number of transfusions given to the patients and their T4/T8 ratios. As shown in Fig 2, it appeared that patients with the highest number of blood transfusions were the ones with the lowest T4/T8 ratios. We can at present give no explanation for this, but it is tempting to suggest that immunosuppressive agents (microbiologic or nonmicrobiologic) are transferred during blood transfusions. Whether this (or these) agent is related to the ones purportedly demonstrated in patients with acquired immunodeficiencies18 remains to be established. Another possibility could be that these changes are caused by the transfer of cytomegalovirus (CMV) with the transfusions, but since these were not screened for CMV, we have not been able to address this question.

To a certain degree, our findings contrast to those of Anderson et al who found a mean T4/T8 ratio of 1.8.16 However, as previously mentioned, many of these patients do not fit into the more strict criteria for primary preleukemia,1 and these studies are therefore not readily comparable with ours.

When evaluating the T cell component using pan-T MoAb (Fig 1), an increased percentage of T cells was demonstrated in some of the patients, who also exhibited lowered percentages of B cells, as evaluated by the MoAb anti-B1. These data could suggest that the increased numbers of T8+ lymphocytes exert suppression on the B lymphocyte lineage, but final proof of this awaits functional studies of the in vitro immunoglobulin secretion in these patients, and such are now in progress. It is, however, interesting to note that Anderson et al16 using anti-immunoglobulin antisera in three preleukemic patients did not find a similar decrease in the number of B cells. These authors did, however, observe a lack of Epstein-Barr virus receptors on B lymphocytes in their patients, and based on this finding it was hypothesized that the B cells in the preleukemia patients might have a deficiency in mature immunocompetent B cells.

The natural history of the development in the single patient from a protracted stage of RA through RAEB to AML is only partly elucidated, and obviously disease parameters of predictive value regarding malignant transformation are highly sought after since these patients are difficult to treat once they have reached the AML stage. With this in mind, the finding of greatest potential interest that emerged from our study is the increased numbers of immature myeloid cells in the bone marrows that reacted with the antibodies anti-My7 and to a lesser extent anti-My9 since this adds considerably to our knowledge of the use of antmyeloid MoAbs in myeloid differentiation disorders. This finding is best seen in light of our comparative studies (Fig 5) that clearly demonstrated that progression from the initial RA stage to RAEB could not be predicted by bone marrow morphology. In contrast, patients with high numbers of My7+ cells (irrespective of morphology) seemed to be the ones susceptible to RAEB progression. In connection with this, it should be noted that progression to RAEB was always accompanied by an increase in My7+ cells in the bone marrow. However, the My7 determinations shown in Fig 5 are all from the time of diagnosis.

The immunologic heterogeneity observed in our patients might be useful in the longitudinal studies performed for the purpose of prognostication and demonstration of changes indicating a transformation to an overt AML. Regarding this, it is noteworthy that during a short observation period (less than 6 months) the antigen expression remained stable, thus demonstrating the validity of these assays (Table 2). The apparent asynchrony between My7 expression and morphology thus indicates that immunologic phenotyping in RA patients should be included in the diagnostic armature. Naturally, My7 is of primary importance in this context, but it might well be envisaged that an immaturity index of a given bone marrow might be constructed that could yield even more information. This might—apart from My7—include HLA-DR antigens that are known to be present on
large proportions of AML cells as well as a mature myeloid antigen, eg, NAT9, that, in contrast to My8, is not expressed by monocytes. Consequently, the combined presence of My7 and HLA-DR (AAS-1) and a decrease in NAT-9 expression might be more predictive than My7 alone. Construction of such ratios might possibly also be of importance in monitoring the response to treatment of AML patients.

Our understanding of the immune response against early neoplasias has been drastically altered during the recent years. Earlier assumptions that educated cytotoxic T lymphocytes might kill emerging malignant clones have not been fulfilled, and it is now believed that the NK cell is a more likely candidate for cell-mediated lysis of malignant cells. We have shown earlier that preleukemic patients are deficient in NK function, and here we extend this conclusion by demonstrating decreased numbers of cells with NK function and antibody dependent killer activity (hemopoietic dysplasia). Leuk Res 6: 175, 1982

Mal studies in patients with the primary preleukemic syndrome (HTLV-III) awaits either semisolid agar experiments or immunoperoxidase staining of bone marrow sections with relevant MoAbs.

In conclusion, we have demonstrated a number of abnormalities in the distribution of leukocyte subsets from blood and bone marrow in primary preleukemic patients. Of these, the decreased T4/T8 ratio in the blood and the increased proportion of cells in the bone marrow possessing the immature myeloid antigen My7 might be of importance in the pathogenesis and for the diagnosis of this potentially prema-

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