The Phenotype of the Neoplastic Cells of Hairy Cell Leukemia Studied With Monoclonal Antibodies

By Jan Jansen, Tucker W. LeBien, and John H. Kersey

Eighteen cases of hairy cell leukemia were studied with a battery of polyclonal anti-Ig and nine monoclonal antibodies to determine the lineage of the hairy cells (HC) and the stage of their maturation arrest. Hairy cells tend to nonspecifically bind many antisera and precautions had to be taken to avoid nonspecific fluorescence of the cells.

All but one case was reactive with one light chain type and one or more heavy chain isotype antisera as reported before. All cases studied were positive for monomorphic HLA-DR determinants, using monoclonal antibody 7.2. Most cases tested (6/7) were positive with the B-lineage related antibody PI 153/3. While most cases (15/18) were nonreactive with the B-lineage related antibody BA-1, they became positive (5/5) following in vitro culture. Seven out of nine cases were reactive with OKM1. Common acute lymphoblastic leukemia antigen (CALLA) was absent in all (15) cases tested and the ALL associated structure p24/BA-2 was absent from 16 of 18 cases. HC from none of the cases were clearly positive with the T-cell antibodies 9.6., or TA-1, whereas in only 1/18 the cells reacted with T101.

The results of this study support the B cell lineage of most HC, and show the presence of multiple phenotypes. In combination with the surface Ig present on the cells, a hierarchy of phenotypes is postulated, with Sig^-^, BA-1, PI 153/3, HLA-DR being the most immature, and Sig^B^, BA-1, PI 153/3, HLA-DR the most mature.

The origin of the neoplastic cells of hairy cell leukemia (HCL, leukemic reticuloendotheliosis) has been frequently debated in recent years. Most investigators, however, now agree that hairy cells (HC) most likely belong to the B-lymphocyte lineage. More specifically, several groups have found indications that the HC show a maturation arrest at a more mature stage than the cells of chronic lymphocytic leukemia (CLL). Thus the HC often show surface Ig of multiple isotypes, and sometimes cytoplasmic Ig is present. Occasionally, the cells produce an M-protein.

The introduction of monoclonal antibodies has provided an invaluable tool to study the maturation arrest of neoplastic cells and to categorize these cells within a certain hematopoietic lineage. A variety of monoclonal antibodies are currently being used to assist in the classification and diagnosis of human leukemias. Thus, some monoclonal antibodies react with cells primarily of T cell lineage, while others are directed against antigens that are present at certain stages of B cell development, but lost as B cells mature into plasma cells. The antibody does not bind to monocytes or T cells, but does bind weakly to mature myeloid cells.

BA-2 recognizes a 24,000 dalton cell surface protein (p24) on early normal and neoplastic cells of primarily B cell, but also T peripheral blood of mononuclear cells with hairy cytoplasmic protrusions. In every case the HC showed acid phosphatase activity that was resistant to preincubation with tartrate. The diagnosis was confirmed in every case by the histological examination of the spleen and/or a bone-marrow biopsy. We studied only samples that contained > 75% HC. Most of the samples had been stored in liquid nitrogen in the presence of 10% DMSO, and were rapidly thawed just prior to use.

Antibodies

The monoclonal antibodies utilized in this study have all been described previously, and were used as diluted ascitic fluid at saturating binding conditions. As controls for nonspecific binding, we used ascitic fluid obtained from mice injected with unfused myeloma cells ('control ascitic fluid,' CAF), and in some instances an IgM mouse myeloma protein (MOPC 104E, Litton Bionetics, Kensington, Md). Both were used at a concentration equal to the least diluted monoclonal antibody. Additionally, a control for the second stage of the indirect immunofluorescence method was used. The following monoclonal antibodies were studied:

- BA-1 recognizes an antigen on cells at multiple stages of B cell development, but lost as B cells mature into plasma cells.
- BA-2 recognizes a 24,000 dalton cell surface protein (p24) on early normal and neoplastic cells of primarily B cell, but also T

MATERIALS AND METHODS

Patients

The diagnosis of HCL in the 18 patients studied had been suggested by the clinical picture, and by the presence in the peripheral blood of mononuclear cells with hairy cytoplasmic protrusions. In every case the HC showed acid phosphatase activity that was resistant to preincubation with tartrate. The diagnosis was confirmed in every case by the histological examination of the spleen and/or a bone-marrow biopsy. We studied only samples that contained > 75% HC. Most of the samples had been stored in liquid nitrogen in the presence of 10% DMSO, and were rapidly thawed just prior to use.

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cell lineage. The antibody does not bind to mature B or T cells.

- PI 153/3 an antibody raised against neuroblastoma cells, also binds to early and mature B cells. However, neoplastic plasma cells do not react with PI 153/3. This antibody was kindly supplied by Dr. R. Kennett (Philadelphia).
- Monomorphic HLA-DR (or Ia-like) determinants were studied with monoclonal antibody 7.2, kindly provided by Dr. J. A. Hansen (Seattle).
- 9.6., an antibody that reacts with all mature T cells and binds to the receptor for sheep erythrocytes, was also a kind gift from Dr. Hansen.
- TA-1 reacts with T lymphocytes and monocytes.
- T101, a pan T cell antibody that reacts additionally with the cells from patients with surface Ig positive CLL, was kindly provided by Dr. I. Royston (San Diego).
- OKM1, an antibody that reacts with monocytes, myeloid cells, and subpopulations of E-rosette positive cells, was obtained from Ortho Pharmaceuticals (Raritan, NJ).
- Finally, J5, a monoclonal antibody that recognizes the common ALL antigen (CALLA) was a kind gift from Drs. J. Ritz and S. F. Schlossman (Boston).

For the recognition of surface Ig (S1g) we used antibodies against Fab and the various heavy and light chain specificities raised in goat, sheep, or rabbit. These antibodies had been conjugated with tetramethyl-rhodamine-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) and have been described before.

**Indirect Immunofluorescence**

Binding of monoclonal antibodies was studied with the indirect immunofluorescence method. Fifty lambda of cells (@ 15 x 10^6/ml) were incubated with 50 lambda of antibody at 4°C for 30 min. Then the cells were washed twice, and incubated with an FITC labeled goat antimouse globulin (FITC-GAMG; Meloy Laboratories, Springfield, Va.) for another 30 min at 4°C. The cells were washed again and the cell pellet, resuspended in a minimal amount of medium, was deposited into a small drop of 90% buffered glycerol on a cover slip. With minimal pressure an objective slide was applied and the cover slip was sealed with nail polish. The slides were studied with a Zeiss fluorescence microscope equipped with epifluorescence condensor IV F1, mercury light source HBO 50W, combinations of filters for narrow band excitation and emission, and a 63/0.80 Ph 2 objective. At least 200 cells were examined per slide. Since HC are easily recognizable in phase contrast microscopy, fluorescence of HC and nonhairy cells could be documented separately. Specimens were considered to be positive when >15% of the HC were stained. Additional studies were done using the FACS IV (Becton & Dickinson, Mountain View, Ca.). The relative fluorescence intensity of 40,000 cells was determined per sample.

**Cell Cultures**

HC were cultured at 1 x 10^6/ml in RPMI 1640 containing 20% fetal bovine serum. The cells were maintained in T flasks (Falcon 3013; growth area 25 cm²) for up to 4 days at 37°C in a humidified atmosphere of 5% CO₂.

**RESULTS**

**Background Staining**

Cells from patients with HCL are known to avidly bind antimmunoglobulin antisera, possibly through the receptor for the Fc portion of IgG. Therefore, great care had to be taken to avoid false positive reactions, resulting from the nonspecific binding of either the FITC-GAMG or the monoclonal antibodies. Indeed, the HC of most patients showed detectable fluorescence with FITC-GAMG alone, or with CAF plus FITC-GAMG (Fig. 1). The fluorescence pattern with these controls varied from very dim to bright. In only occasional cases were these controls completely negative. To reduce the background staining the cells had to be cultured overnight at 37°C,
stored overnight at 4°C, or fixed with diluted formaldehyde. No single method was effective in all cases, but in each case we succeeded in reducing the background with at least one of the three methods. We considered the results with the monoclonal antibodies to be unreliable unless CAF at the same concentration as the least diluted monoclonal antibody plus FITC–GAMG showed no fluorescence or only a very faint rim (‘ghosts’). To further define the specificity of binding, every positive reaction was reevaluated following culture for 24 hr at 37°C. When the binding was positive again, the antigen recognized by the particular antibody was considered to be present on the HC.

Surface Immunoglobulins

The SIg phenotypes of most of these patients have been described in detail elsewhere. The HC were studied after fixation with diluted formaldehyde, which has been shown to remove the nonspecifically bound immunoglobulins from the cell membrane. The HC of five patients had γ isotypes, five had αγ, three had μδ, and two each had αγδ and αδ. In one case no SIg could be detected. The light chain pattern was always monoclonal; eight cases had κ light chains and nine λ light chains.

Monoclonal Antibodies

The data obtained from the 18 patients studied are listed in Table 1. The antigen recognized with monoclonal antibody BA–1 was found in only three out of 18 cases (17%). The fluorescence pattern in these three patients was moderately bright. BA–2 bound to >15% of the cells in only two out of 18 patients (11%). The intensity of fluorescence in these cases was brighter than found in patients with CLL. Monoclonal antibody PI 153/3 reacted with the HC of six out of seven cases studied (86%); the fluorescence intensity varied from very weak to moderately bright. HLA–DR monomorphic determinants, as detected with monoclonal antibody 7.2, were present on the HC of all 18 patients studied. The intensity of fluorescence varied, but in general was bright.

The anti–T cell antibodies usually only recognized small, nonhaired, cells. The population of nonhaired cells in both spleen and peripheral blood almost exclusively consisted of these T cells. The data in Table 1 indicate that in most cases a close correlation was found between the proportions of fresh cells positive with 9.6, TA–1, and T101. However, in cases one and two, a larger percentage of cells reacted with T101 than with TA–1 or 9.6. In case two at least part of the HC reacted with T101 and continued to do so after overnight culture at 37°C. On two occasions (cases 10 and 13) many freshly thawed HC were positive for 9.6. but negative for TA–1 and T101. Furthermore, these cells did not form E rosettes. After overnight culture only the small lymphoid cells were positive for 9.6. suggesting that the pattern obtained with fresh cells was another example of nonspecific binding by HC. Therefore, the percentages of 9.6. positive cells after overnight culture are listed for cases ten and thirteen. The HC in 7 out of 9 cases (78%) bound the OKM1 antibody and continued to do so after 4 days of culture.

**Table 1. Phenotypes of the neoplastic cells of 18 patients with hairy cell leukemia, as determined with monoclonal antibodies and antihuman immunoglobulin reagents.**

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<th>Specimen</th>
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<th>9.6</th>
<th>TA–1</th>
<th>T101</th>
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*As determined with anti-polyvalent antiserum. pb = peripheral blood; sp = spleen; nd = not done.
The common ALL antigen, detected with monoclonal antibody J5, seemed to be present on small proportions of fresh cells of some patients, but was never demonstrable after overnight culture at 37°C or storage at 4°C.

Effect of Culture on Cellular Phenotype

Culture of HC for up to four days did not change the morphology of the cells significantly. In most cases the cells transiently adhered to the flask, but they could always easily be recovered by repeated flushings. The percentage viable cells left after 4 days of culture was 77 ± 7 (mean ± SE). In none of the five cases studied was any evidence of proliferation found, as indicated by cell numbers and the absence of mitotic figures in cytocentrifuge slides stained with Wright's stain. As shown in table 2, the cultured cells continued to be positive for SIg and with the monoclonal antibodies they had reacted with at day 0. The intensity of fluorescence usually remained unchanged, but sometimes increased with 7.2. In the five cases shown in table 2, the HC that had been BA-I at day 0 (7 ± 2%, mean ± SE) converted to a BA-I phenotype following two days of culture (64 ± 7% of cells positive for BA-I at day 2; Fig. 2). Interestingly, by day 4 many of the HC reverted to a BA-I phenotype (13 ± 8% positive cells). The HC at day 2 did not bind the IgM mouse myeloma protein MOPC 104 E, suggesting that the binding of BA-I was not due to receptors for the Fc part of IgM, present on the cells of many cases of HCL.

DISCUSSION

The concept that HC are neoplastic B cells has been developed on the basis of intrinsic SIg on the cells of various antibodies. Table 2. Effect of in vitro Culture on Reactivity of the Neoplastic Cells of 5 HCL Patients with Antiimmunoglobulin Reagents and Various Antibodies.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Day</th>
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In this series SIg was present in 17 out of 18 cases and was always monoclonal with respect to light chain expression. Additional arguments for a B cell origin of HC are the presence of intracytoplasmic Ig in a minority of cases,1,3,23 Ig production by HC in vitro and/or in vivo,1,4,23 formation of mouse rosettes by HC,23 and immunoglobulin gene rearrangement (29, Dr. S. J. Korsmeyer, personal communication). The results in this study obtained with monoclonal antibodies support this B cell origin. The presence of HLA-DR determinants, previously documented with polyclonal antisera,30,31 and now confirmed with monoclonal antibody 7.2., and the presence of the antigens reacting with antibodies BA-I most of the cases.1,4,21
and BA−2 in minorities of cases and antibody PI 153/3 in the majority of cases,15 all fit the B cell concept. Previous studies had already demonstrated that the cells of most cases of HCL do not form E rosettes or react with polyclonal anti-T cell antisera.2,3 The data reported here on the absence of antigens recognized by the anti-T cell antibodies 9.6., TA-1, and T101, confirm the non-T cell origin of most cases of HCL. A few cases of HCL with T cell features or with combined B and T cell features have been reported.31,32 None are included in the present series. The cells of the majority of patients studied reacted with monoclonal antibody OKM1. This reactivity could be interpreted as an argument in favor of a monocytic origin of HCL.

Alternatively, these cases of HCL may originate from a rare population of OKM1+B−lymphocytes, resulting in hybrid cells with both monocytic and B−lymphocytic features.2,3 However, OKM1 is certainly not specific for monocytes. Myeloid cells and T cells with FcIgG receptors also react with this antibody,19,34 and we have found a case of B−prolymphocytic leukemia to be positive (J. Jansen, unpublished observation). In addition, the lack of reactivity of HC with TA-1 is evidence against a monocytic phenotype in this disease. Nevertheless, the HC of these and other cases deserve study with other monocyte specific antibodies to solve this matter.

On the basis of the results obtained in this study several major phenotype groups may be distinguished. Cases one through three belong to the “immature” group in which the cells are SIg or SIgκ or SIgλ and BA−1+, BA−2+, PI 153/3+, HLA-DR+. In at least one of these cases the HC also reacted with T101. Thus, these cells may have a maturation arrest at a stage similar to the cells of the majority of cases of CLL. In the latter disease the cells are SIg+ or SIgκ or SIgλ−δ, BA−1+, BA−2−/−, PI 153/3−, HLA-DR−, and T101−.11−17 However, the most “mature” cases of CLL with an M protein in the serum are T101+.11 Cases four through sixteen differed somewhat in that they had a more “mature” phenotype, i.e., BA−1−, BA−2+/−, PI 153/3−, HLA-DR+. The SIg in this group was mostly γ or αγ. Another phenotype SIgκγ, BA−1−, BA−2−, PI 153/3−, HLA-DR+ is represented by patient 17. This may represent a maturation arrest at the most mature stage, where the antigen recognized by PI153/3 is lost. Different phenotypes have also been reported on the basis of membrane proteins in polyacrylamide gel electrophoresis,35 and it would be interesting to see how these various subgroups correlate. On the basis of the results in HCL patients, we postulate a maturation scheme of B cell differentiation as shown in Fig. 3. In this scheme HCL should obviously be located between CLL and myeloma, confirming previous reports that HC are more mature B lymphocytes than the cells of CLL.1,3,23

Culture of HC for up to four days did not result in significant changes in morphology, SIg phenotype, and phenotype using most monoclonal antibodies tested. No significant proliferation was observed during the culture period. The only consistent change in surface phenotype was found using BA-1 in which all five cases studied changed from BA−1− to BA−1+. The nature of this change is not clear, but could relate to changes in the status of cellular differentiation or to nutrients added to the culture medium. Further studies are currently underway to evaluate phenotype changes induced under various in vitro conditions.

ACKNOWLEDGMENT

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