

Heterogeneous CD52 Expression among Hematologic Neoplasms: Implications for the Use of Alemtuzumab (CAMPATH-1H)

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Abstract Purpose: CD52 is a GPI-linked glycoprotein expressed by B cells, T cells, monocytes, and macrophages. The humanized monoclonal antibody alemtuzumab (CAMPATH-1H) is specific for CD52 and is Food and Drug Administration – approved for the treatment of relapsed or refractory chronic lymphocytic leukemia (CLL). The utility of CAMPATH in the treatment of other hematologic neoplasms has been explored; however, a comprehensive survey of CD52 expression among a broad spectrum of WHO-defined tumor types has not been completed.

Experimental Design: We evaluated 294 hematologic neoplasms for the presence of CD52 using standard immunohistochemical techniques on paraffin-embedded biopsy specimens fixed with formalin, B-Plus, Zenker's acetic acid, or B5-formalin.

Results: The vast majority of low-grade B cell lymphoproliferative disorders (CLL/small lymphocytic leukemia, follicular lymphoma, lymphoplasmacytic lymphoma, hairy cell leukemia, and mucosa-associated lymphoid tissue lymphomas) express CD52. In addition, we found that the majority of precursor B cell acute lymphoblastic leukemia/lymphomas express this antigen. In contrast, there is surprising heterogeneity in CD52 expression among more aggressive B cell lymphomas, with 25% of cases of diffuse large B cell lymphoma and Burkitt lymphoma demonstrating no detectable CD52. In addition, the majority of neoplasms of the T cell lineage are negative for the antigen, including most cases of precursor T cell acute lymphoblastic leukemia/lymphoma, anaplastic large cell lymphoma, and peripheral T cell lymphoma, not otherwise specified. Finally, the vast majority of cases of acute myeloid leukemia, Hodgkin lymphoma, and multiple myeloma are negative for CD52 expression.

Conclusion: In contrast with CLL, the variable expression of CD52 among other hematologic malignancies suggests that target validation on a case-by-case basis will likely be necessary to guide the rational analysis of CAMPATH therapy.

CD52 is a 21 kDa cell surface glycoprotein of unknown function that is expressed by B and T lymphocytes, natural killer (NK) cells, monocytes, macrophages, and some dendritic cells, but not by granulocytes, RBC, platelets, or hematopoietic progenitor cells (1, 2). In the 1980s, rodent-derived monoclonal antibodies specific for CD52 were among the first to be "humanized" by transferring the antigen-specific, complementary determining regions to a human framework (2, 3). The most successful of these is CAMPATH-1H (alemtuzumab), a humanized IgG₁ monoclonal antibody. When bound to cell surface CD52, CAMPATH-1H induces lysis via activation of complement and direct cell-mediated cytotoxicity (4, 5).

CAMPATH-1H is currently approved for the treatment of patients with relapsed/refractory chronic lymphocytic leukemia (CLL) who have failed prior fludarabine-based chemotherapy (6, 7). Because of the marked lymphopenia resulting from CAMPATH therapy, the antibody has also been used to prevent graft versus host disease following allogeneic bone marrow transplantation (8–11). Recently, several studies have evaluated CAMPATH treatment of additional hematolymphoid malignancies including peripheral T cell lymphoma (PTCL), T cell prolymphocytic leukemia, and cutaneous T cell lymphoma (12–16). Although a small number of patients were examined in these studies, the drug had antitumor activity but also substantial toxicity. These findings emphasize the importance of limiting CAMPATH therapy to those patients most likely to show an antitumor response.

Despite the interest in using CAMPATH to treat lymphoid malignancies, there is no comprehensive examination of CD52 expression in specific diseases. This may be ascribed, in part, to the difficulty of evaluating the expression of CD52 in archived tissues (17). Herein, we report methods for the detection of CD52 on fixed paraffin-embedded tissue specimens and analyze CD52 expression on an extensive series of hematologic neoplasms including current candidates for CAMPATH therapy.

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Materials and Methods

Case selection

Two hundred and ninety-one of the 294 cases were derived from the case files of the Department of Pathology at Brigham and Women's Hospital (Boston, MA), with institutional review board approval. Three cases were derived from the files of Genzyme Genetics (New York, NY). Paraffin-embedded tissue sections were available for all 294 cases. In addition, 23 cases of lymphoplasmacytic lymphoma (LPL) from Brigham and Women's Hospital had fresh tissue for flow cytometric analysis, as did all of the cases from Genzyme Genetics. Specifically, specimens consisted of whole tissue sections reflecting a combination of bone marrow biopsies, excisional biopsies of lymphoid tissue, and in a few cases, splenectomies. Tumor subclassification was based on the WHO criteria for hematologic malignancies using a combination of morphologic, immunohistochemical, flow immunophenotypic, and when necessary [i.e., Burkitt lymphoma, acute myeloid leukemia with inversion 16 (AML-inv(16))], cytogenetic studies (18). The diagnoses were confirmed by review of the original pathology report that summarizes the morphologic and ancillary study findings and by re-review of stained paraffin sections by two hematopathologists (S.J. Rodig and J.L. Kutok). Any case for which the available information and/or tissue was not sufficient for a confident diagnosis was not included in the study.

Immunodetection of CD52

Immunohistochemistry. Five-micron-thick formalin-, B-Plus-, B5-formalin-, or Zenker's acetic acid-fixed paraffin-embedded tissue sections were immunostained. For formalin-fixed or B-Plus tissues only, slides were pretreated with 0.25% trypsin (Sigma, St. Louis, MO) at 37°C for 20 minutes. All slides were reacted with Peroxidase Block (DAKO, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity. Primary rat anti-human CD52 antibody with the same complementary determining regions as the human antibody (clone YTH34.5; Serotec, Oxford, United Kingdom) was applied (formalin and B-Plus = 1 µg/mL; B5-formalin = 125 ng/mL; Zenker's acetic acid = 100 ng/mL) for 1 hour at room temperature. Slides were then washed in 50 mmol/L of Tris-Cl and 0.05% Tween 20 (pH 7.4).

For formalin-, B-Plus-, and B5-formalin-fixed tissue, a two-step method was used to detect CD52 expression. Rabbit anti-rat secondary antibody (DAKO) was applied at a dilution of 1:750 for 30 minutes, followed by incubation with anti-rabbit, horseradish peroxidase-conjugated antibody solution (Envision plus, DAKO) for 30 minutes. Immunoperoxidase staining was developed using a diaminobenzidine chromogen (DAKO) according to the manufacturer's instructions, and slides were counterstained with Harris hematoxylin. For Zenker's acetic acid-fixed tissue only, a single-step detection method was sufficient to detect CD52 after primary antibody incubation, slides were incubated with anti-murine, horseradish peroxidase-conjugated antibody solution (Envision plus, DAKO) for 30 minutes and then developed with diaminobenzidine as above.

For frozen tissue, slides were fixed in cold acetone, and rat anti-human CD52 antibody was used at 1 µg/mL and detected with 1:750 rabbit anti-rat secondary antibody (DAKO) for 30 minutes, followed by anti-rabbit, horseradish peroxidase-conjugated antibody solution (Envision plus, DAKO) for 30 minutes. In cases with available frozen tissue, the expression of CD52 was also evaluated using a murine monoclonal antibody (clone HI186, 500 ng/mL; Serotec) recognizing an epitope distinct from the rat monoclonal antibody. This was detected with anti-murine, horseradish peroxidase-conjugated antibody solution (Envision plus, DAKO) for 30 minutes and then developed as above. HI186 was not adequate for staining paraffin-embedded specimens.

Flow cytometry. For the cases of LPL analyzed by flow immunophenotyping at Brigham and Women's Hospital, mononuclear cell preparations of fresh bone marrow aspirates were stained with FITC-conjugated rat anti-human CD52 (clone YTH34.5; Serotec) and

analyzed on a FACScalibur flow cytometer using CellQuest V.3.3 software (BD, Franklin Lakes, NJ). Three cases derived from Genzyme Genetics [precursor-B acute lymphoblastic leukemia/lymphomas (pre-B ALL), AML-inv(16), and PTCL-not otherwise specified (NOS)] were previously analyzed by flow immunophenotyping at the referring institution using a murine monoclonal antibody recognizing human CD52 (clone CF1D12) from Caltag/Invitrogen (Carlsbad, CA).

Analysis of CD52 expression

Reactivity for CD52 was determined and scored independently by two hematopathologists (S.J. Rodig and J.L. Kutok). Tissue samples evaluated for CD52 expression were considered positive if the tumor showed unequivocal positive staining in the majority of tumor cells. In the vast majority of positive cases, the tumor cells showed strong staining of the cytoplasm and the cell membrane. Nuclear staining was seen in very rare cases, and was considered nonspecific with the cases excluded from the study. For cases in which the tumor was negative for CD52, strong positive CD52 staining among reactive small lymphocytes was required as an internal control. For 23 cases of LPL, there was concurrent flow cytometric evaluation for CD52 expression among the lymphoid populations. In each case, positive CD52 expression as determined by immunohistochemical staining of the biopsy specimen was confirmed by positive staining using flow immunophenotyping (data not shown). In addition, flow cytometric data for CD52 expression was available for one case of precursor-B ALL, one case of AML-inv(16), and one case of PTCL-NOS derived from the case files of Genzyme Genetics. The two former cases were positive for CD52 expression by flow analysis, whereas the latter case was negative for CD52 expression. In each case, the flow cytometric findings correlated with the results of immunohistochemical staining for CD52 on paraffin-embedded tissue sections (data not shown).

Results

As expected, immunostaining of formalin-fixed paraffin-embedded reactive tonsil for CD52 revealed broad expression of the antigen among lymphoid cells, including those within the B cell-rich follicles and the T cell-rich interfollicular regions (Fig. 1A). Similarly, within reactive Zenker's acetic acid-fixed and decalcified, paraffin-embedded bone marrow sections, scattered interstitial lymphocytes showed robust membrane expression of CD52 (Fig. 1B). In contrast, the nonlymphoid cells within the marrow, including early and maturing erythroids, granulocytic precursors, and megakaryocytes, were found to be negative for the antigen.

B-Cell lymphoproliferative diseases. The examination of 88 indolent B cell lymphoid malignancies [CLL/small lymphocytic leukemia (SLL), follicular lymphoma, hairy cell leukemia, LPL, extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)] in Zenker's acetic acid, B5, B-Plus, or formalin-fixed paraffin-embedded material revealed that the vast majority of these tumors were positive for CD52 (Table 1). Within this cohort, 100% of CLL/SLLs, the Food and Drug Administration-approved target of CAMPATH therapy, expressed CD52 (Fig. 1C). In cases of SLL, we repeatedly observed higher levels of CD52 expression in the prolymphocytes and paraimmunoblasts within proliferation centers than in the small lymphocytes outside proliferation centers. Almost all cases of follicular lymphoma, hairy cell leukemia, LPL, and MALT lymphoma were also CD52 positive (Table 1).

Although CD52 was broadly expressed on indolent B-cell lymphoproliferative disorders, with the vast majority of tumor cells expressing the antigen, select tumors with extensive

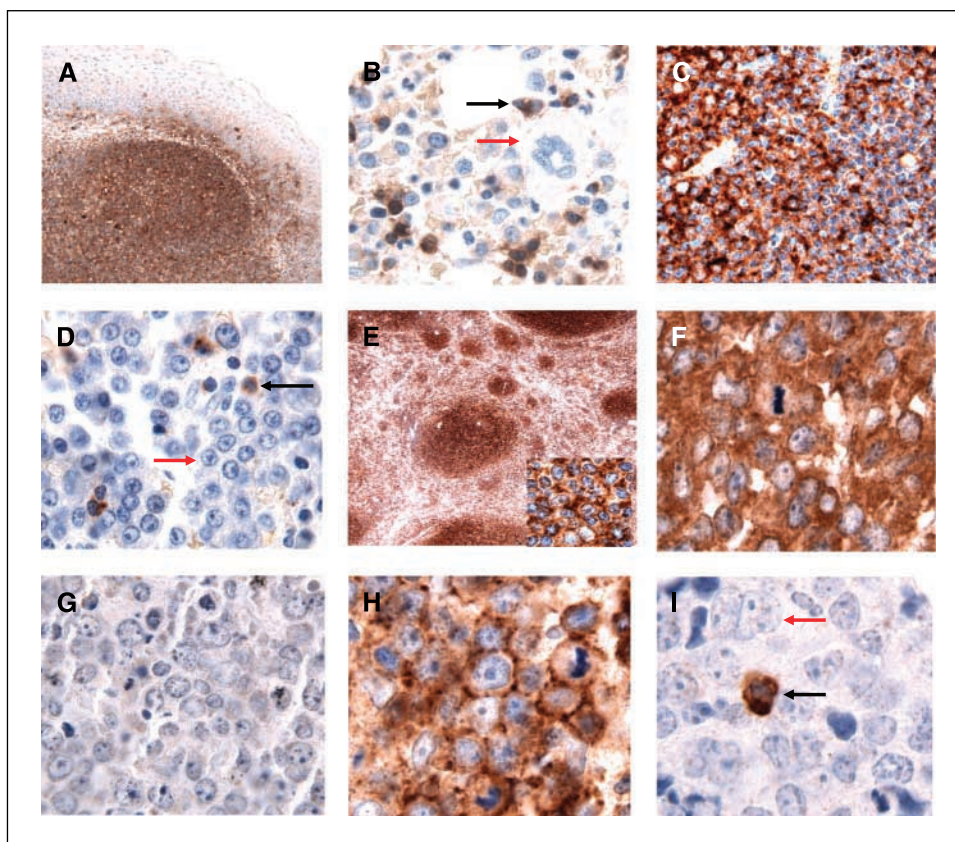


Fig. 1. Immunohistochemical staining for CD52 in B cell lymphoproliferative disorders. *A*, tonsil, formalin-fixed tissue section (magnification, $\times 40$). *B*, normal bone marrow; Zenker's acetic acid – fixed tissue section (red arrow, megakaryocyte; black arrow, reactive lymphocyte; magnification, $\times 1,000$). *C*, SLL; lymph node, formalin-fixed tissue section (left, the edge of a proliferation center; magnification, $\times 1,000$). *D*, multiple myeloma; bone marrow, Zenker's acetic acid – fixed tissue section (red arrow, malignant plasma cell; black arrow, reactive lymphocyte; magnification, $\times 1,000$). *E*, mantle cell lymphoma; lymph node, B5-formalin – fixed tissue section (magnification, $\times 40$; inset, $\times 1,000$). *F* and *G*, diffuse large B cell lymphomas; lymph nodes, B5-formalin – fixed tissue sections (*F*, positive staining tumor; *G*, negative staining tumor; magnification, $\times 1,000$). *H* and *I*, Burkitt lymphomas; lymph nodes, B5-formalin – fixed tissue sections (*H*, positive staining tumor; *I*, negative staining tumor; red arrow, tumor cell; black arrow, reactive lymphocyte; magnification, $\times 1,000$).

plasmacytic differentiation showed low to absent expression of the antigen within the plasmacytic component. This finding was noted both among the LPL and MALT lymphomas (Table 1). Nevertheless, the B-lymphocytes of both LPL and MALT lymphomas were consistently positive for CD52. Twenty-three of 27 cases of LPL also had concurrent flow immunophenotypic analysis done. For each case, the expression of CD52 was detected on the malignant B lymphoid population by flow immunophenotyping in agreement with the results of the immunohistochemical stain (data not shown). Consistent with the lack of expression of CD52 on fully differentiated plasma cells, 22 of the 23 cases of multiple myeloma were negative for CD52 (Fig. 1D).

We next examined more aggressive B cell tumors including mantle cell lymphoma (Fig. 1E), diffuse large B cell lymphoma, and Burkitt lymphoma (Fig. 1F-I; Table 1). Whereas all mantle cell lymphomas were CD52 positive, 25% of diffuse large B cell lymphomas and Burkitt lymphomas lacked detectable CD52 expression (Fig. 1G-I). These data indicate that the target of CAMPATH therapy is more variably expressed in these aggressive B cell malignancies and raise the possibility that the drug may be useful for only a select subset of patients. Finally, none of the examined Hodgkin lymphomas were CD52 positive (Table 1).

T-Cell lymphoproliferative diseases. Neoplasms of mature T lymphocytes and NK cells include anaplastic large cell lymphoma, NK cell leukemia/lymphoma, angioimmunoblastic T cell lymphoma, hepatosplenic T cell lymphoma, adult T cell leukemia, and PTCL-NOS. These tumors, which are much less common than B cell malignancies, are often poorly responsive

to conventional chemotherapy. As a result, there is considerable interest in the potential utility of CAMPATH for their treatment. However, in contrast to mature B cell tumors, we found that a much smaller percentage of mature T cell malignancies are positive for CD52.

Specifically, all examined anaplastic large cell lymphomas (Fig. 2A) and the majority of NK and NK/T cell lymphomas, angioimmunoblastic T cell lymphomas, hepatosplenic T cell lymphomas, and PTCL-NOS were CD52 negative (Fig. 2B-E; Table 1). Among PTCL-NOS, only 35% of the examined tumors expressed detectable levels of the antigen (Fig. 2D-E), highlighting the heterogeneity of CD52 expression inherent within this diagnosis. In contrast, all examined adult T cell leukemias were CD52 positive (Fig. 2F).

Because we did not detect CD52 expression on a substantial subset of mature T cell lymphomas fixed and embedded in paraffin, we considered the possibility that our detection method may not have been robust enough to identify low levels of CD52 expression that may be seen on fresh, or unfixed, frozen tissue. To address this possibility, in 18 cases, we compared the results of using paraffin-embedded tissue with paired frozen tissue from the same tumor immunostained with either the rat monoclonal antibody (clone YTH34.5; Serotec) or a murine monoclonal antibody that recognizes a distinct epitope of CD52 (clone HI186, Serotec). These cases were evenly divided between those with robust staining for CD52 and those without detectable staining for CD52 in the tumor cell component on paraffin-embedded tissue. We found a complete concordance of results in both positive and negative staining for CD52 between the paraffin-embedded and frozen

Table 1. CD52 expression across tumor types

Diagnosis	Cases tested (n)	CD52 positive (n)	CD52 positive (%)
CLL/SLL	25	25	100
Follicular lymphoma	9	9	100
Hairy cell leukemia	10	10	100
LPL	27	27	100*
MALT lymphoma	17	14	82 [†]
Mantle cell lymphoma	20	20	100
Burkitt lymphoma	12	9	75
Diffuse large B cell lymphoma	28	21	75
Multiple myeloma	23	1	4
Hodgkin lymphoma	11	0	0
Anaplastic large cell lymphoma	10	0	0
NK and NK/T cell lymphoma	3	1	33 [‡]
Angioimmunoblastic lymphoma	5	2	40
Hepatosplenic; γ/δ T cell lymphoma	6	2	33
PTCL-NOS	20	7	35 [§]
Adult T cell leukemia/lymphoma	5	5	100
Pre-B ALL	19	17	89
Pre-T ALL	14	1	7
AML (total)	30	5	17
Minimally differentiated	3	0	0
Without maturation	3	0	0
With maturation	5	0	0
Acute promyelocytic leukemia	2	0	0
Acute myelomonocytic leukemia	10	5	50
Acute monocytic leukemia	4	0	0
Not otherwise specified	3	0	0

*Two of 27 cases negative for CD52 in the plasmacytic component.

[†]Three negative cases showed extensive plasmacytic differentiation and only minimal lymphoid component.

[‡]Two of two negative cases confirmed on frozen tissue.

[§]Five of five positive cases and six of six negative cases confirmed on frozen tissue.

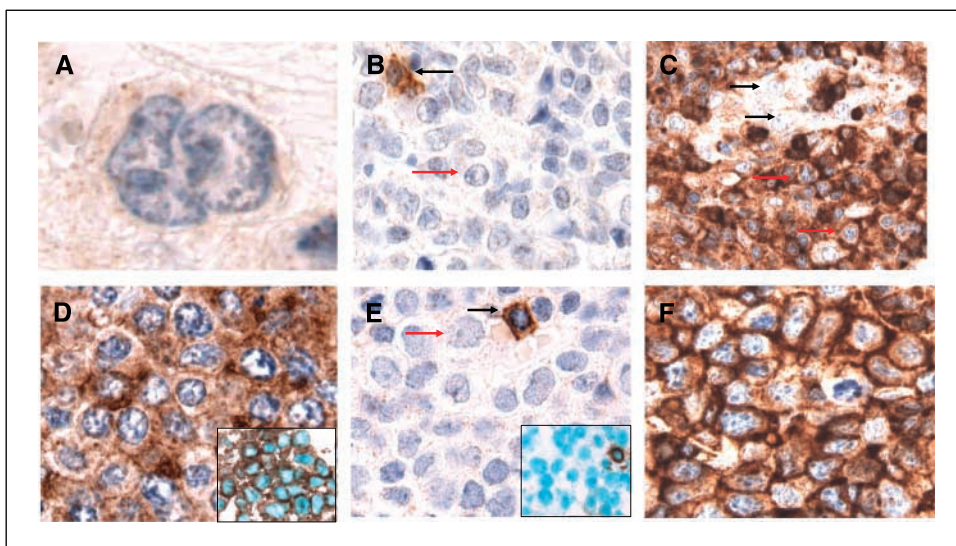
^{||}Four of five positive cases were AML-inv(16) (p13q22).

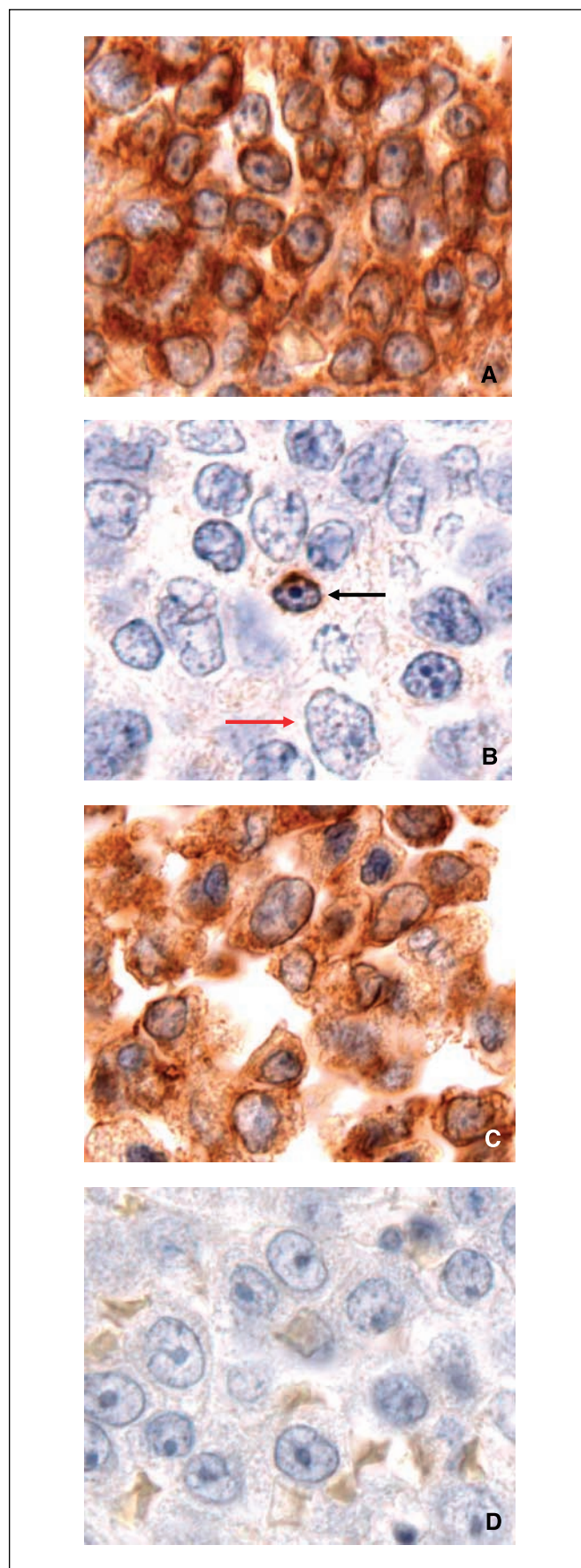
tissues with either of these monoclonal antibodies (Fig. 2D-E, insets; Table 1; data not shown). In addition, for one case of PTCL-NOS, flow cytometric analysis using an additional distinct antibody recognizing CD52 (clone CF1D12; Caltag/Invitrogen) confirmed the results of staining of paraffin-embedded tissue of no detectable antigen expression on the tumor cells (data not shown). Taken together, these findings indicate that CD52 expression is variable among mature T cell

lymphoproliferative disorders, with a majority of these diseases lacking immunohistochemically detectable CD52 expression.

Acute lymphoid and myeloid leukemias. We next evaluated CD52 in acute leukemias. Whereas the majority (89%) of pre-B ALLs were CD52 positive (Fig. 3A), only rare pre-T ALLs (7%) showed CD52 expression (Fig. 3B). For one case of pre-B ALL, flow cytometric analysis was done and confirmed the immunohistochemical staining results of robust expression of CD52

Fig. 2. Immunohistochemical staining for CD52 in T cell lymphoproliferative disorders. *A*, anaplastic large cell lymphoma, lymph node; B5-formalin – fixed tissue section (magnification, $\times 1,000$). *B*, blastic NK cell lymphoma, skin; B plus (red arrow, tumor cell; black arrow, reactive lymphocyte; magnification, $\times 1,000$). *C*, angioimmunoblastic T cell lymphoma, lymph node; B5-formalin – fixed tissue section (red arrow, tumor cells; black arrows, endothelial cells; magnification, $\times 1,000$). *D* and *E*, PTCL-NOS from two separate patients, lymph nodes (*D*, formalin-fixed tissue section; *E*, B5-formalin – fixed tissue section; magnification, $\times 1,000$). *Insets*, the same tumors, frozen tissue immunostained with a second anti-CD52 antibody recognizing a distinct epitope (red arrow, tumor cell; black arrow, reactive lymphocyte; magnification, $\times 1,000$). *F*, adult T cell leukemia/lymphoma; B5-formalin – fixed tissue section (magnification, $\times 1,000$).





by the tumor cells (data not shown). The majority of AMLs (83%) of various subtypes were negative for CD52 (Table 1). However, a subset of acute myelomonocytic leukemias (including four cases classified as acute myelomonocytic leukemia with eosinophilia and the inversion 16) exhibited CD52 expression in the blast population (Fig. 3C). For one case of AML-inv(16), flow cytometric analysis was done and confirmed the immunohistochemical staining results of weakly positive CD52 expression (data not shown). These findings are in contrast with acute monoblastic leukemias, which were CD52 negative (Fig. 3D).

Discussion

We describe a robust immunohistochemical method to detect CD52 in paraffin-embedded biopsy specimens and report on CD52 expression in an extensive series of hematologic neoplasms. Currently, CD52 expression is most commonly evaluated by flow cytometric techniques on fresh specimens or by immunohistochemistry on frozen tissue. Our technique, which can be done on fixed archival material, has the great advantage in that fresh or frozen tumor cells are not required. This allows for CD52 status to be assessed on previously biopsied patient tumors without the need for re-biopsy. Using archived material could be of tremendous value in recruiting patients for clinical trials with CAMPATH, in which CD52 status of the tumor is required. In addition, robust staining using this methodology is seen in tissues fixed with all of the major fixatives and the expression of CD52 can be directly visualized on the morphologically abnormal cell population.

Although we noted the ubiquitous expression of CD52 by indolent B cell lymphoproliferative disorders, there were variable CD52 expressions in the cellular constituents of SLL, and down-regulation of the antigen on plasma cells in tumors with extensive plasmacytic differentiation. This was particularly true in multiple myelomas, which we found to be negative for CD52 in the vast majority of cases—in agreement with reports by other authors using flow cytometric detection techniques (19, 20). In addition, we found that a significant subset (25%) of cases of diffuse large B cell lymphoma and Burkitt lymphoma lack CD52 expression. In contrast to the B cell malignancies, the majority of mature T cell lymphoproliferative disorders, with the apparent exception of adult T cell leukemia/lymphoma, are negative for CD52. Similarly among acute leukemias, CD52 is generally expressed by pre-B ALLs, but is rarely seen in pre-T ALLs. Finally, the Reed-Sternberg cells of Hodgkin lymphoma were negative for CD52 expression in all tested cases.

Our finding that the majority of T cell malignancies are negative for CD52 was surprising given the normal expression of CD52 on peripheral blood T cells. We considered the possibility that the sensitivity of staining paraffin-embedded

Fig. 3. Immunohistochemical staining for CD52 in acute leukemias. *A*, pre-B ALL, bone marrow; Zenker's acetic acid – fixed tissue section (magnification, $\times 1,000$). *B*, pre-T ALL, bone marrow; Zenker's acetic acid – fixed tissue section (*red arrow*, lymphoblast; *black arrow*, reactive lymphocyte; magnification, $\times 1,000$). *C*, acute myelomonocytic leukemia with eosinophilia and the inversion 16, bone marrow; Zenker's acetic acid – fixed tissue section (magnification, $\times 1,000$). *D*, acute monoblastic leukemia, bone marrow; Zenker's acetic acid – fixed tissue section (magnification, $\times 1,000$).

tissue might be lower than with fresh or frozen tissue—leading to false negative results. However, we found complete correlation in results between paraffin-embedded tissue and paired frozen tissue immunostained with either the same or a second, distinct monoclonal antibody specific for CD52. For a subset of cases, flow immunophenotypic data was available that also showed 100% correlation with the immunohistochemical data.

Given the encouraging activity of CAMPATH in the treatment of CLL refractory to fludarabine-based chemotherapy, several pilot studies evaluating CAMPATH in the treatment of other hematologic tumors have been reported. Based on three studies, response rates as high as 44% have been found in patients with a broad array of relapsed/refractory B cell non-Hodgkin lymphomas (21–23). Notably, all responders to CAMPATH had low-grade disease. Several individual responses to CAMPATH have also been reported for patients with relapsed/refractory precursor B-ALL, and these findings have prompted a phase I/II CALGB study which adds CAMPATH to induction chemotherapy in previously untreated B-ALL (24, 25). Finally, a 36% overall response rate has been reported

for the single agent use of CAMPATH in the treatment of patients with relapsed/refractory PTCL (16).

Unfortunately, CD52 expression by the malignant cells was not established in any of these trials prior to therapy. Given our finding that a substantial fraction of aggressive B cell tumors and T cell tumors are negative for CD52, documentation of CD52 antigen expression on the malignant cells may be useful for the proper interpretation of CAMPATH trial results and to identify the patient population with the optimal opportunity for a good clinical response.

This is particularly true given that the encouraging clinical activity of CAMPATH is tempered by the significant incidence of infectious complications. Infections related to impaired T cell immunity have been identified in all reported trials, including cases of cytomegalovirus reactivation, aspergillosis, *Pneumocystis carinii* pneumonia, herpes zoster, mucormycosis, and others. It is this potential for severe infectious complications associated with CAMPATH that reinforces the necessity of targeting the agent only to those patients that are likely to benefit. This widely applicable robust immunostaining method for CD52 may allow for better identification of this patient population.

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