A high rate of *CLL phenotype* lymphocytes in autoimmune hemolytic anemia and immune thrombocytopenic purpura

Minor CLL-like clones are found in ~3% of healthy individuals. AIHA and ITP are common in CLL and may be causally linked. We investigated the presence of *CLL phenotype* lymphocytes in 11 cases of primary AIHA, 18 of ITP and 2 of Evans' Syndrome, compared with 26 age-matched healthy controls. A population of 'CLL phenotype' was seen in 6/31 patients compared to 1/26 healthy controls (χ^2=3.9; p=0.05). Such clones may be important in the pathogenesis of autoimmune blood disorders.

Haematologica. 2008 Jan; 93:(1)151-152. DOI: 10.3324/haematol.11822

Autoimmune hemolytic anemia (AIHA) and immune thrombocytopenic purpura (ITP) are the commonest autoimmune conditions affecting blood. AIHA has an incidence of 1-3 cases per 100,000/year[1] and about 18% of AIHA cases subsequently develop a clinically diagnosed lymphoproliferative disease.[2] ITP has an incidence of 5.8-6.6 per 100,000/year.[3] Conversely, warm AIHA and ITP occur in 4-40% and 1-2% respectively of patients with chronic lymphocytic leukemia (CLL).[4] The association between autoimmune processes and CLL is even more striking, since about a third of CLL patients have a positive DAGT[5] and we have demonstrated previously that CLL cells were the dominant antigen presenting cell (APC) type, activating T cells with auto-antigen in patients with complicating AIHA.[6] By conventional criteria, the lifetime risk of CLL is 0.25%, but more sensitive flow cytometric (FACS) techniques[7] have demonstrated CLL-like clones in approximately 3.5% of a normal population, the prevalence rising with age.[8] Together, these observations suggest the possibility that underlying small lymphoproliferative clones may be important in the pathogenesis of these autoimmune conditions. This hypothesis was tested by seeking *CLL phenotype* lymphocytes in peripheral blood samples from patients with primary AIHA and ITP by FACS. We investigated the presence of *CLL phenotype* lymphocytes in 11 cases of primary AIHA (5 newly diagnosed), 18 cases of ITP (4 newly diagnosed) and 2 patients with Evans' Syndrome. A total of 31 cases (age range=22-87 years, mean=61; SD=19) was therefore compared with 26 age matched healthy controls (age range=24-96 years, mean=62; SD=23) who were admitted for minor surgical procedures.

FACS analysis of peripheral blood lymphocytes was carried out as described by Rawstron *et al.*[8] using 4-color flow cytometry to identify CD19$^+$ B-cells with stronger CD5, weaker CD20 and CD79b phenotype using antibodies from BD Pharmingen (Oxford, UK). A minimum of 200,000 total leukocytes were acquired and data analyzed using CELLQuest Software on a FACS cytometer (Becton-Dickinson, Oxford, UK). An initial region (R1) was set around cells with CD19 expression and low side scatter (granularity). A second region (R2) was set on the basis of the physical characteristics of CD19 positive B-cells. A third gate (R3) was created to exclude cells that were binding equivalent amounts of anti-CD19 and anti-CD5 antibodies non-specifically. Further analyses were performed on cells that fell within all these three regions. Regions R4, R5 and R6 were created to identify the population of B-cells with high CD5 and lower CD20 and CD79b expression. More than 50 cells in each of R4, R5 and R6 gates was regarded as positive (Figure 1). Six out of 31 patients showed *CLL phenotype* B-cells with more than 50 cells in each of gates R4, R5 and R6 (Figure 2), while only 1 out of 26 healthy controls was positive (χ^2=3.9; p=0.05). Of the 6 positive patients, 3 were drawn from the 11 patients with primary AIHA and 3 from the 18 patients with ITP. Of the 2 patients with Evans' syndrome, neither was positive. Out of 6 positive cases, 2 had newly diagnosed AIHA and 1 had newly diagnosed ITP prior to any treatment. All 3 of the other patients, 1 with AIHA and two with ITP, were being treated with steroids at the time of sampling.

It remains unconfirmed whether clonal B-cells are associated with idiopathic ITP or AIHA. In this study, we report a higher prevalence of *CLL phenotype* B lymphocytes in these patients compared to an age-matched control group. However, we may have underestimated the prevalence of these cells since many of these patients were on immunosuppressive therapy at the time of sampling, and this may have reduced their number. We used the criteria proposed by Rawstron *et al.*,[8] but did not formally prove clonality. It is possible, but not proven, that the lymphocytes that we detected are important in the pathogenesis of the disease. We speculate that these *CLL phenotype* cells may process antigen differently from conventional APCs, therefore

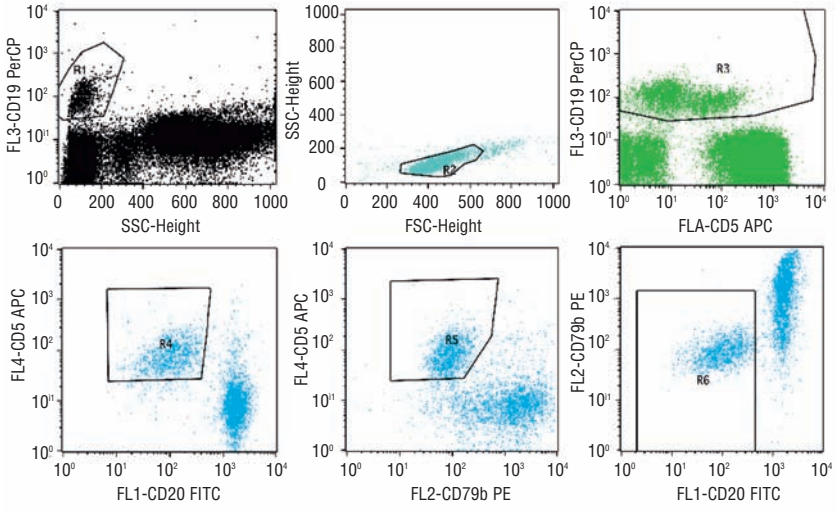

Figure 1. The gating strategy for detecting 'CLL phenotype' cells.

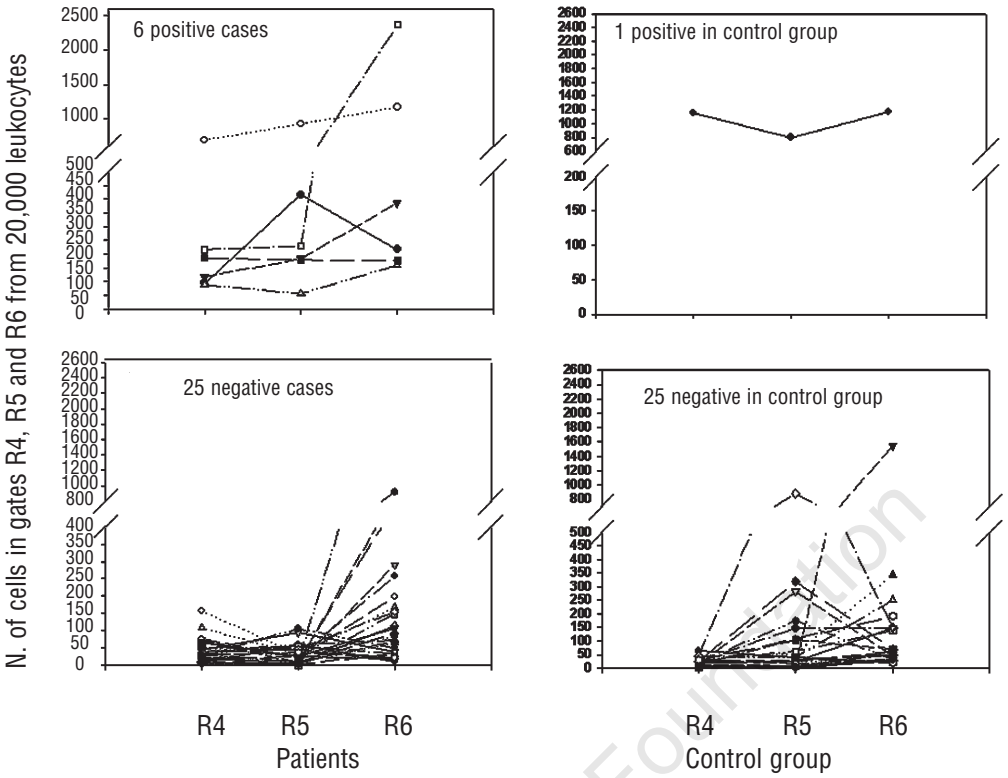

Figure 2. ***CLL phenotype*** **cells in patients vs. healthy controls. Presence of significant** ***CLL phenotype*** **cells was defined as more than 50 cells in each of R4, R5 and R6 gates in patients with AIHA and ITP (n= 6) and control (n= 1).**

revealing *cryptic* epitopes that are not subject to previously established tolerance and so cause autoimmunity. Future research, involving identification of clonal B-cell population, using 6-color FACS or Ig gene mutation status with PCR in newly diagnosed AIHA and ITP prior to any treatment should clarify the relevance of our findings.

Sajjan Mittal,[1] Morgan G. Blaylock,[2] Dominic J. Culligan,[1] Robert N. Barker,[2] Mark A. Vickers[2]

[1]Department of Clinical Haematology, Aberdeen Royal Infirmary, Aberdeen; [2]Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK

Funding: this project was supported by the NHS Grampian endowment fund and 'Friends of Anchor' charitable fund.

Key words: autoimmune hemolytic anemia, immune thrombocytopenic purpura, clonal B-cells, 'CLL-phenotype' lymphocytes.

Correspondence: M.A. Vickers, Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen AB25 2ZD, UK. E-mail: m.a.vickers@abdn.ac.uk

References

1. Bottiger LE, Westerholm B. Acquired haemolytic anaemia. I. Incidence and aetiology. Acta Med Scand 1973;193:223-6.
2. Sallah S, Wan JY, Hanrahan LR. Future development of lymphoproliferative disorders in patients with autoimmune hemolytic anemia. Clin Cancer Res 2001;7:791-4.
3. McMillan R. Chronic idiopathic thrombocytopenic purpura. N Engl J Med 1981;304:1135-47.
4. Hamblin, T. Autoimmune disease and its management in chronic lymphocytic leukemia. In: Cheson BD, ed. Chronic Lymphoid Leukemias. New York: Marcel Dekker, 2001. p. 435-58.
5. Barcellini W, Montesano R, Clerici G, Zaninoni A, Imperiali FG, Calori R, et al. In vitro production of anti-RBC antibodies and cytokines in chronic lymphocytic leukemia. Am J Hematol 2002;71:177-83.
6. Hall AM, Vickers MA, McLeod E, Barker RN. Rh autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B cells. Blood 2005;105:2007-15.
7. Rawstron AC, Kennedy B, Evans PA, Davies FE, Richards SJ, Haynes AP, et al. Quantitation of minimal disease levels in chronic lymphocytic leukemia using a sensitive flow cytometric assay improves the prediction of outcome and can be used to optimize therapy. Blood 2001;98:29-35.
8. Rawstron AC, Green MJ, Kuzmicki A, Kennedy B, Fenton JA, Evans PA, et al. Monoclonal B lymphocytes with the characteristics of "indolent" chronic lymphocytic leukemia are present in 3.5% of adults with normal blood counts. Blood 2002;100:635-9.