Abstract
EDTA induced clumping of lymphoid cells, both benign and malignant, in peripheral blood samples has been reported only rarely. Such clustering presents the laboratory and pathologist with unique difficulties in the accurate diagnosis of these disorders. A case of low grade B cell splenic lymphoma is presented with lymphocyte clumping in smears made from EDTA anticoagulated peripheral blood, the fourth case described in which neoplastic lymphoid cells form clusters in vitro in peripheral blood. The association with splenic lymphomas (three of the four cases) is intriguing but its significance remains uncertain.

Keywords: EDTA; leucoagglutination; lymphocytes; lymphoma

Methods
Smears made from blood collected in tubes containing 5.4 mg K2EDTA and 0.129 M sodium citrate (Becton Dickinson Vacutainer Systems) were stained by Wright’s method and examined by light microscopy. Bone marrow aspirate smears were stained by the Wright-Giemsa method. All samples of peripheral blood collected were evaluated by a Coulter STKS haematology analyser. Flow cytometric evaluation of the peripheral blood was performed with the Coulter Epics-Profile II flow cytometer. Antibodies to B and T cell markers (Becton Dickinson) and immunoglobulin heavy and light chains (Dako) were used to characterise the phenotype of the circulating lymphocytes.

Case report
A 65 year old white woman with a 25 year history of polycythaemia vera and stable splenomegaly presented with symptoms of increasing spleen size and subsequently underwent splenectomy. Preoperative examination of the peripheral blood showed a white count of 5.5 x 10^9/litre, with 62% neutrophils and 27% lymphocytes; no clusters of lymphocytes were identified.

Two days after surgery, a routine complete blood count showed marked leucocytosis (36 x 10^9/litre). The automated differential count showed 85% neutrophils and 5% lymphocytes; however, a manual differential on a peripheral smear from the same sample showed 40% neutrophils and 42% lymphocytes. Microscopically, prominent clustering of lymphocytes was observed, with groups ranging from 2 to 15 cells involving approximately 13% of the total lymphocytes. There was similar clustering when the patient’s blood was drawn into sodium citrate.
phocyte field on the scattergram plots (fig 1, right), with a corresponding decrease in lymphocyte agglutination on the peripheral smear.

Immunophenotypic analysis of the lymphocytes in the peripheral blood showed a population of B cells comprising 85% of total lymphocytes, which marked with antibodies to CD11c, CD19, CD20, CD22, IgD, and IgM. Lambda light chain restriction was observed. The cells were negative for CD5, CD10, CD23, CD24, CD25, and CD56.

The postoperative bone marrow biopsy showed a hypercellular marrow for age (90–95%) due to erythroid and megakaryocytic hyperplasia. Small CD20 positive lymphocytes were scattered uniformly throughout the marrow, with no distinct lymphoid infiltrates or clusters. The bone marrow aspirate buffy coat smears contained several clusters of lymphocytes similar to those seen in the peripheral blood, which were interpreted as contamination from peripheral blood.

Although the morphological and immunophenotypic features of this process did not fit precisely into a diagnostic category, it was felt that it was a low grade B cell lymphoproliferative disorder most closely resembling SLVL.

The patient was discharged home thereafter without complications, and remained asymptomatic, receiving no chemotherapy. Periodic peripheral blood examinations continued to show inaccurate automated leucocyte differential counts and lymphocyte clustering on peripheral smears until 20 months after splenectomy, with total leucocyte counts of 14–15 × 10⁹/litre. Her latest complete blood count, nearly 30 months postoperatively, showed an automated white cell count of 12.7 × 10⁹/litre with occasional lymphocyte and lymphocyte-neutrophil agglutination in EDTA collected peripheral blood.

Discussion

EDTA associated agglutination in peripheral blood is not uncommon for platelets but has also been reported in neutrophils, benign and malignant lymphocytes, and, rarely, in multiple cell lines. The exact mechanism underlying this phenomenon has not been determined. Cold agglutinins (IgM) have been implicated in platelet satellitosis and aggregation, and may play a role in leucoaggregation as well, as evidenced by the reduction in aggregates after warming or anti-IgM treatment. In this case, warming reduced agglutination. In platelets, EDTA is believed to alter the structure of cell membrane proteins at low temperatures, uncovering antigenic determinants which interact with EDTA dependent platelet agglutinating autoantibodies (predominantly IgM) to result in agglutination. A similar mechanism has been postulated in other cell types; additionally, arachidonic acid, leukotriene B4, and adrenaline (epinephrine) have been implicated in lymphocyte aggregation.

The phenomenon typically disappears as the patient recovers. Importantly, these changes occur only in the laboratory; there is no evidence of in vivo agglutination or pathological effects resulting from cell clustering.

The clustering of malignant lymphocytes is rare, with only three cases reported. Bizzaro and Piazza first reported the phenomenon involving a case of chronic lymphocytic leukaemia (CLL); two cases of SLVL with malignant circulating lymphocytes showing agglutination were subsequently reported. Deol and colleagues found two cases of benign lymphocytes showing EDTA induced aggregation, one patient having a urinary tract infection, the other (an HIV positive individual) a B cell lymphoma without bloodstream involvement. Spontaneous lymphocyte aggregation in patients with extremely high (more than 400 × 10⁹/litre) lymphocyte counts has also been described. It is interesting to note that with this case, SLVL occurred in three of the four reported cases of agglutination of neoplastic lymphocytes.
It is in the use of automated cell analysers that the presence of circulating lymphocyte clusters is most problematic. The aggregates show increased size and complexity relative to individual lymphocytes. Analysers using cell size and complexity for automated white blood cell differential counts misinterpret the aggregates as large granulocytes, resulting in artificially increased granulocyte counts and undercounted lymphocytes. Instruments which perform the differential by plotting size versus peroxidase show the presence of cell aggregates by a band in the region corresponding to large size and low peroxidase—that is, adjacent to the monocyte-basophil area. Many automated cell analysers do not have warning flags for cell groups, further compounding the difficulty. It is possible that this phenomenon occurs more often than is currently realised.

Several measures are available to reduce or eliminate cell aggregation in peripheral blood specimens. Using heparin or sodium citrate as the anticoagulant, immediately smearing a freshly drawn, non-anticoagulated specimen, and heating the tube before smearing have all been reported to be useful. Interestingly, Deol et al reported slight clumping in a heparinised specimen on visual examination of the peripheral smear. In our case, sodium citrate did not appreciably change the clustering tendency of the lymphocytes, while heating significantly reduced clumping both in the analyser and on the smear.

One final difficulty encountered regarding this phenomenon is determining the identity of the clustered cells. Carcinoma metastasising to the bone marrow has rarely resulted in circulating clusters of tumour cells. In contrast to leucocyte aggregation, malignant epithelial cell clusters within the peripheral blood (carcinocythaemia) is a true in vivo event, independent of anticoagulation or manipulation of the sample, and occurs in the setting of pancytopenia secondary to end stage bone marrow replacement. In difficult cases, flow cytometry or immunostaining techniques may be of benefit.

In conclusion, we have reported a fourth case of neoplastic lymphocytes forming clusters in peripheral blood and the third such case to occur in a spleen based low grade B cell lymphoma. The association with splenic lymphomas is intriguing, but its significance remains uncertain.

Splenic B cell lymphoma with lymphocyte clusters in peripheral blood smears

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doi: 10.1136/jcp.53.3.228

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