December 1998 (Volume 39, Number 4)

Splenic Lymphoma with Villous Lymphocytes Should Be Included in the Differential Diagnosis of Massive Splenomegaly

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Aim. To distinguish between patients with reactive lymphocytosis and those with malignant lymphoid proliferations, with particular reference to hyper-reactive malarial splenomegaly and splenic lymphoma with villous lymphocytes.
Patients. Forty-four patients, residents of the Ashanti region of Ghana that is hyperendemic for Plasmodium falciparum malaria, were studied. All patients had splenomegaly greater than 10 cm. They were given proguanil 100 mg/day for a minimum of 6 months. Lymphocyte surface phenotypes were studied on the peripheral blood smears, immunoglobulin gene rearrangement by the Southern blot technique, serum IgM concentration using the Nor-Partigen-IgM kit, and serum paraprotein concentration by electrophoresis.
Results. Based on the response to proguanil, the patients were categorized into good respondents, partial respondents, and non respondents. Peripheral blood lymphocytes exceeded 30% in 19, and villous lymphocytes were less than 30% in 25 patients.
Conclusion. Splenic lymphoma with villous lymphocytes may be difficult to differentiate from the African variant of chronic lymphocytic leukemia which is associated with splenomegaly and from hyperreactive malarial splenomegaly with lymphocytosis. In West Africa, a peripheral blood lymphocyte count greater than 10 x 109/L, with more than 30% of villous lymphocytes and failure of splenic regression with anti-malarial therapy suggest a diagnosis of splenic lymphoma with villous lymphocytes.

Key words: Ghana; leukemia; leukemia, lymphocytic, chronic; lymphoma; lymphocytosis; malaria; Plasmodium falciparum; splenomegaly

Enlarged spleen measuring 10 cm or more from the left subcostal margin to its tip is a common clinical finding among adults in West Africa (1). In general, the diagnosis of most of these disorders does not present a problem. In West Africa, chronic lymphocytic leukemia (CLL) and hyperreactive malarial splenomegaly are both lymphoproliferative disorders which may present with splenomegaly and lymphocytosis and are therefore clinically difficult to differentiate (2).

Chronic Lymphocytic Leukemia

CLL is a malignant clonal disorder characterized by the production and accumulation of functionally defective lymphocytes (3). In the West, CLL is a disease of the elderly with a male to female ratio of 2:1. Patients present with recurrent infections and lymphadenopathy. A peripheral blood smear which shows an increased number of small mature lymphocytes that express B cell markers and CD5 antigen on their surface is consistent with a diagnosis of CLL (4). In the West African environment, the dominant presenting feature of CLL is splenomegaly rather than lymphadenopathy (5). In a study of CLL among Congolese Bantus, two distinct disease patterns emerged (6): one corresponded to the classical description of CLL with lymphadenopathy, modest splenomegaly, and grossly elevated white blood cell (WBC) count, whereas the second group was characterized by massive splenomegaly, moderately increased leukocyte count (4-50x109/L), and hyper- gammaglobulinemia with monoclonal proteins.

Hyperreactive Malarial Splenomegaly

A diagnosis of hyperreactive malarial spleno- megaly is usually considered if there is a massive splenomegaly for which no locally prevalent cause can be found. It occurs in long term residents of malarious areas and is characterized by high serum IgM levels and hepatic sinusoidal lymphocytosis (7). Although parasitemia is infrequent (8), splenic enlargement regresses slowly with a prolonged anti-malarial treatment (9). The maximum response may take up to 1 year to be achieved, and the spleen will re-enlarge if the treatment is ceased.
Hyperreactive malarial splenomegaly is thought to result from repeated attacks of malaria which lead, by a process that is not well understood, to the production of lymphocytotoxic antibodies specific for

suppressor T lymphocytes. These are involved in the regulation of IgM production by B lymphocytes. The consequent over-production of IgM results in macromolecular immune complexes, the clearance of which results in hypertrophy of mononuclear phagocyte system and massive hepatosplenomegaly (10,11).
In West Africa, hyperreactive malarial spleno- megaly may be associated with a high peripheral blood lymphocyte count in 10% of the patients, making the condition difficult to differentiate from African chronic lymphocytic leukemia (12).
Splenic Lymphoma with Villous Lymphocytes
Prior to our study, the only reports of splenic lymphoma with villous lymphocytes were from the industrialized countries. Splenic lymphoma with villous lymphocytes in the West is characterized by splenomegaly in 85% of the patients and the presence of a significant number of characteristic lymphocytes with uni- or bi-polar villous projections in the peripheral blood (see Fig. 1). The male to female ratio is 1.77:1, with a mean age at diagnosis of 68.4 years (range 42-89). The WBC count is usually above 10x109/L, with the majority of cells consisting of lymphocytes, of which over 30% have villous projections (13). Monoclonal protein bands are present in the serum and/or urine of about two thirds of the patients. The cells are positive for the cell marker CD19, thus confirming their B cell lineage.

Figure 1: Villous lymphocytes in a Ghannian patient shown by light microscopy. [view this figure]

Patients and Methods
Patient Selection and Follow-up
The study group comprised 44 Ghanaian patients living in the Ashanti region of Ghana where malaria is holoendemic. All the patients were referred to the hematology department between 1986-1992 for the investigation of massive splenomegaly. Schistosomiasis, cirrhosis, myelofibrosis, and chronic granulocytic leukemia were excluded. All the patients had spleens extending from 8 to 41 cm (mean 16.8) below the costal margin. There were 35 women and 9 men aged between 15 and 75 years (mean 36.4). Follow-up was performed monthly and the adopted strategies included providing free proguanil (Paludine, Zeneca Limited, Macclesfield, Cheshire, UK) seeing patients in a quiet room outside the main out-patient department, stressing the need for drug compliance, and consultations free of charge provided for the patients' relatives. Response to the treatment was assessed by monthly measuring the size of the spleen.
Hematological Examinations
Hemoglobin examination, total and differential white cell counts with careful assessment of lymphocyte morphology were performed on the Wright-stained peripheral blood smears.
Immunological Studies
Lymphocyte surface phenotypes. Monoclonal antibodies were used in the alkaline phosphatase anti-alkaline phosphatase technique (APAAP) to assess the percentage of B and T lymphocytes and T cell subsets in the peripheral blood smears (14). Blood smears were air dried, wrapped in aluminium foil, and frozen unfixed at -20 °C for storage. Prior to processing, the slides were allowed to thaw at room temperature. They were then fixed in methanol-acetone-formaldehyde and washed in TRIS-buffered saline. Slides were sequentially incubated with monoclonal antibody, rabbit anti-mouse antibody, alkaline phosphatase anti-alkaline phosphatase for 30 minutes each, and washed in between each application with TRIS-buffered saline. The second and third incubating steps were repeated for 10 minutes each. Substrate mix was applied for 30 minutes, and the slides were washed in distilled water and placed in filtered Harris hematoxylin. The stain was washed off in tap water and the slides mounted and viewed under oil immersion. Positively stained lymphocytes appeared red (Fig. 2). The percentage of positively stained villous lymphocytes was assessed by counting the number of positively stained villous lymphocytes out of a hundred positive lymphocytes. The counting was done under oil immersion. Monoclonal antibodies used in the first layer of the alkaline phosphatase anti-alkaline phosphatase technique were CD2 (pan-T cell marker), CD4 (helper and inducer T cell marker), CD5 (pan-T marker; B-CLL lymphocyte marker), CD8 (cytotoxic/supressor T cell marker), CD19 (pan B cell marker), and k and l immunoglobulin light chain markers.

Figure 2: Villous lymphocytes positive with an antibody to CD19 antigen. [view this figure]

Serum IgM measurement. Serum IgM concentrations were measured using a NOR-Partigen-IgM kit (Behring, Marburg, Germany) based on the principle of radial immunodiffusion. Ten mL of serum were placed into the wells. A series of standard sera were used to construct the reference curve and a

control sample was included in each plate. The plates were left for two days in a humidified atmosphere at 37 °C to allow the IgM to diffuse into the gel and the diameters of the precipitates were measured. The corresponding IgM concentrations were determined from the standard curve.
Serum electrophoresis for detection of paraproteins. Serum electrophoresis was carried out using cellulose acetate membrane with a TRIS-Na barbital buffer, pH 8.8. Serum samples were applied to a presoaked cellulose acetate membrane and electrophoresed at 150 V for 50 minutes. Normal samples were included in each run. The membranes were stained with Ponceau S (Gelman Sciences, Ann Arbour, Mich, USA) and cleared in 5% acetic acid. Any abnormal bands in the patients' sera were typed by immunofixation. This involved electrophoresis as above, allowed by a small piece of cellulose acetate membrane in the diluted antiserum and placing it on the electrophoresis strip at the position of the abnormal band. Electrophoresis was continued for 5 min at 150 V to allow the anti-serum to react with the paraprotein. The small piece of cellulose acetate membrane was rinsed off under running water and the membrane washed twice in 0.9% saline for 10 min before staining with Ponceau S for 5 min and decolorizing in acetic acid for 10 min.

DNA Analysis

Cells from 20 mL of EDTA anticoagulated blood were lysed with a cell lysis buffer (3 mL 1 mol/L TRIS, 32.9 g sucrose, 1.5 mL 1 mol/L magnesium chloride, 3 mL Triton X-100, and water added to make a total volume of 300 mL). Pelleted nuclei were suspended in 2 mL nuclear lysis buffer (50 g guanidine thiocyanate, 0.5 g sodium N-lauryl sarcosine, 2.5 mL sodium citrate pH 7.0, 0.7 mL 2-mercaptoethanol, and water added to make up a total volume of 100 mL). The samples were stored at 40 °C before being transported to the UK for DNA purification (15).
DNA was purified by isopycnic banding through cesium chloride. DNA samples were digested with at least two specific restriction endonucleases and the fragments were separated by electrophoresis through a 0.8% agarose gel. Separated DNA fragments were denatured and transferred from the gel onto a nylon filter by the Southern blot technique (16). Radioacively-labelled gene probe (JH) (Amersham International, Amersham, UK) was hybridized in hybridization buffer overnight to the DNA on the filter which was then washed in three increasing stringent solutions: 2X sodium chloride and sodium citrate solution (SSC)/0.1% sodium dodecylsulphate solution (SDS); 1X SSC/0.1% SDS; 0.1X SSC/0.1% SDS at 65 °C for a total of 1 hour, to remove unbound probe. The filter was autoradiographed for 4-7 days and the patterns of DNA fragments examined. The presence of a single band in addition to the germ line configuration indicated the presence of a monoclonal population of lymphocytes.

Results

Clinical Features

The majority of the patients included in this study were asymptomatic and had their splenomegaly detected incidentally, often during the course of outpatient care. Only 16 out of 44 patients spontaneously complained of splenomegaly. Other symptoms in a minority of patients included fever, yellow eyes (jaundice), and symptoms of anemia such as tiredness, shortness of breath or palpitations. Some presented with more than one symptom. On examination, all patients had splenomegaly and 41/44 also had hepatomegaly 1 to 14 cm. Four out of 44 patients had jaundice on admission.

Hematological Findings

Forty-four patients were categorized into two groups because the diagnosis of splenic lymphoma with villous lymphocytes requires the presence of at least 30% of circulating villous lymphocytes. The first group comprised 19 patients (14 women and 5 men) with more than 30% of villous lymphocytes and the second group comprised 25 patients (21 women and 4 men) with less than 30% of villous lymphocytes. The age of the first group ranged from 15 to 75 years, with a mean of 45.5 years, whereas for the second group, it ranged from 15 to 65 years, mean 29.1 years. Patients with more than 30% of villous lymphocytes were significantly older than those who had less than 30% of villous lymphocytes ($p=0.0057$).
The mean hemoglobin was 7.6 g/dL (3.5-10.8) for those with more than 30% of villous lymphocytes and 7.8 g/dL (2.8-15.5) for those with less than 30%. The mean WBC was 42.7 x 109/L (1.7-93 x 109/L) for the first group and 5.2 x 109/L (1.8-14) for the second patient group, respectively.

Response to Treatment

All the patients were available for a follow up for a minimum period of 6 months. The response to treatment was based on the reduction in the spleen size after the treatment with proguanil, 100 mg/day. We defined 3 grades of response to treatment (Table 1): 1. Good response: >40% reduction in the splenic size which was sustained by the treatment; 2. Partial response: 15-40 % reduction in the splenic size or recurrence of splenomegaly while on treatment; 3. No response: <15 % reduction

in the splenic size despite at least 3 months of treatment. According to these criteria, out of 44 patients, 23 showed good response, 10 partial, and 11 no response to therapy.

Table 1: Response to treatment with proguanil in 44 patients with splenomegaly . [view this table]

Protein Electrophoresis

One patient in the group with more than 30% of villous lymphocytes had two small paraprotein bands typed as IgM-k and IgD-k. Although 5 other patients in that group had monoclonal bands, these could not be typed because of their relatively low intensity compared to the background level of immunoglobulins. All the patients in the group with less than 30% of villous lymphocytes had a polyclonal increase in g-globulin compared to the local Ghanaian controls (20 healthy blood donors).

Lymphocyte Surface Markers

The results of the lymphocyte phenotype studies were expressed as the percentage of total lymphocytes which were positive with each monoclonal antibody (Table 2).

Table 2: Peripheral blood lymphocyte phenotype in 44 patients with splenomegaly [view this table]

The group with more than 30% of villous lymphocytes had less CD2+ T cells (p£0.001) and more B cells (p<0.001) than the group with less than 30% of villous lymphocytes, although the CD4:CD8 ratio was similar for the each group. The number of CD5+ cells in each group reflected the total number of T cells (i.e., was similar to CD2+ cells). There was therefore no evidence of a separate population of CD5+ cells which were also CD19+ as would be expected in chronic lymphocytic leukemia (17). Lymphocyte clonality studies of immunoglobulin light chains showed restriction to the lambda chain in 3 patients and to the kappa chain in a patient with peripheral blood lymphocyte count over 10 x 109/L in the group with more than 30% of villous lymphocytes. Kappa and lambda monoclonal antibodies were also found in 10 patients, but the results in 5/10 of patients were difficult to interpret due to the heavy background staining by non-specific immunoglobulins. This technical obstacle could be overcome by using cytocentrifuged preparations of washed cells but this facility is not widely available in West Africa.

IgM Studies

The mean IgM concentrations for the group with more than 30% and less then 30% of villous lymphocytes were 5.1 g/L (range 0.1-11.4) and 4.5 g/L (range 0.2-11.1), respectively. They did not differ significantly from each other but were significantly elevated (p=0.0029) compared to local normal controls (mean 1.8 g/L) from 20 healthy blood donors.

DNA Analysis

Five out of 10 tested patients from the group with more than 30% of villous lymphocytes and 1 out of 8 patients from the group with less than 30% had their immunoglobulin heavy chain gene rearranged, indicating the presence of a monoclonal population of lymphocytes.

Previous studies have shown that, in order to make a diagnosis of splenic lymphoma with villous lymphocytes, not only should patients have over 30% of villous lymphocytes, but these cells should also be clonal, i.e., derived from a single cell which has undergone neoplastic change (14). Therefore, in all the patients with villous lymphocytes over 30% who also had evidence of lymphocyte monoclonality splenic lymphoma with villous lymphocytes could be diagnosed. This subdivision of the patients with more than 30% of villous lymphocytes is shown in Table 3. No patients with the white blood counts less than 10x109/L from that group had evidence of monoclonal lymphocytes.

These two sub-types of the patients with more than 30% of villous lymphocytes also showed clear differences in their response to treatment (no patient with a high white count having a good treatment response) and in their lymphocyte phenotype. In those with a high WBC count, B cells predominated, whereas in those with lower WBC counts, most lymphocytes carried T cell markers (p<0.001) (Table 3).

Table 3: Comparison of the findingsa in patients with splenomegaly who had >30% of villous lymphocytes in the peripheral blood with respect to their high and low peripheral blood lymphocyte count. [view this table]

Table 4: Comparison between hyperreactive malarial splenomegaly (HMS) and splenic lymphoma with villous lymphocytes (SLVL) [view this table]

Discussion

Differential Diagnosis of Massive Splenomegaly

We studied clinical, morphological, immunological, and molecular characteristics of 44 patients with massive splenomegaly which was not caused by cirrhosis, chronic myeloid leukemia, sickle cell anemia, schistosomiasis mansoni, or myelofibrosis. Based on the response to the treatment and lymphocyte morphology and clonality, the patients could be clearly divided into three distinct diagnostic groups.

Group 1 – Hyperreactive Malarial Splenomegaly. This group comprises patients with a good and sustained response to treatment, and with polyclonal lymphocytes. Although 6 patients with hyperreactive malarial splenomegaly also had villous lymphocytes exceeding 30%, these cells were not monoclonal and so the presence of these cells alone does not support the diagnosis of lymphoma.

Group 2 – Splenic Lymphoma with Villous Lymphocytes. These patients did not respond to anti-malarial treatment. They had more than 30% or more monoclonal villous lymphocytes and a peripheral blood lymphocyte count of more than 10x109/L.

Group 3 – Intermediate Group. Four patients with 30% of villous lymphocytes had polyclonal lymphocytes, but were partially or wholly resistant to anti-malarial therapy. There could be many reasons for this, including inadequate treatment due to poor compliance or proguanil resistance. However, it is possible that recent advances in the detection of small populations of malignant lymphocytes (e.g., by polymerase chain reaction) could demonstrate the presence of lymphoma cells in some of these patients who would then be classified as those having splenic lymphoma with villous lymphocytes. One patient in this group, a 40-year-old female, had normal white blood count, less than 10% of villous lymphocytes, and a partial response to proguanil. Morphology of her peripheral blood lymphocytes was compatible with a diagnosis of follicular B-cell non-Hodgkin's lymphoma. Such patients have a degree of immune suppression (18) and may be more susceptible to malaria. Prophylaxis with proguanil would therefore cause a slight reduction in the splenomegaly by reducing the level of malaria infection.

Other patients in the group 3 (24%) had heterogeneous features and it was not possible to classify them as hyperreactive malarial splenomegaly, splenic lymphoma with villous lymphocytes or some other form of lymphoproliferative disorder.

Comparison Between Splenic Lymphoma with Villous Lymphocytes in Ghana and the West

Splenic lymphoma with villous lymphocytes has been described almost exclusively in the Western Europeans, predominantly in males with the mean age 68.4 years (range 42-89) at diagnosis (13). The main physical sign was splenomegaly and a monoclonal protein in the serum or urine of 66% of the patients (13). In Ghana, splenic lymphoma with villous lymphocytes affects a younger age group (mean age 36.4 years, range 13-75) with a predominance of women (19). About 20% of our patients had a monoclonal protein in their serum. This is likely to be an underestimate because high levels of background immunoglobulins (which are not found in the residents of the areas with temperate climates) mask the presence of small paraproteins. In addition, some patients only may have a urinary paraprotein (13) and urine samples were not available in our patients.

Splenic lymphoma with villous lymphocytes and hyperreactive malarial splenomegaly are both lymphoproliferative disorders that present with massive splenomegaly, and hyperreactive malarial splenomegaly may be associated in 10% of patients with a high peripheral blood lymphocyte count (12). Thus, hyperreactive malarial splenomegaly may be difficult to differentiate from splenic lymphoma with villous lymphocytes. The most helpful differentiating features were the white blood cell count range of 13.4-82x109/L in splenic lymphoma with villous lymphocytes and <10x109/L in hyperreactive malarial splenomegaly, the response to anti-malarial therapy (poor in splenic lymphoma with villous lymphocytes, good in hyperreactive malarial splenomegaly, and evidence for lymphocyte monoclonality (such as light chain restriction or immunoglobulin gene rearrangements) in splenic lymphoma with villous lymphocytes. In addition, 30% of circulating lymphocytes in splenic lymphoma with villous lymphocytes should have villous morphology and they should carry B-cell surface markers (i.e., CD19+) (20). The presence of a paraprotein also supports the diagnosis of splenic lymphoma with villous lymphocytes (13). Elevated IgM levels, classically cited as essential for the diagnosis of hyperreactive malarial splenomegaly (7), were not helpful in discriminating this disorder from splenic lymphoma with villous lymphocytes in our patients (Table 4).

None of our patients had marked generalized lymphadenopathy or CD5+B lymphocytes and thus did not fulfill the criteria for the diagnosis of classical chronic lymphocytic leukemia (4).

Association Between Hyperreactive Malarial Splenomegaly and Splenic Lymphoma with Villous Lymphocytes

Our study demonstrated a spectrum of diseases from benign polyclonal treatment-responsive hyperreactive malarial splenomegaly to a malignant lymphoma (splenic lymphoma with villous

lymphocytes) resistant to anti-malarial treatment. Some patients had features of both disorders and could not be classified. The presence of >30% of villous lymphocytes in some treatment-responsive patients and the similar clinical and demographic features in both disorders led us to the postulate that, in some cases, hyperreactive malarial splenomegaly may evolve into splenic lymphoma with villous lymphocytes. This was supported by the case of our 45-year-old Ghanaian patient with hyperreactive malarial splenomegaly and 36% of villous lymphocytes who initially had a good response to proguanil (reduction in spleen size from 17 to 4 cm over 7 months). After a year of continuous treatment, her spleen began to increase in size despite continuing proguanil, and her villous lymphocytes elevated to 54% before she was lost to follow-up.

The relationship between hyperreactive malarial splenomegaly and splenic lymphoma with villous lymphocytes may be analogous to that which occurs in endemic Burkitt's lymphoma: repeated B cell stimulation from malaria challenge results in an expanded pool of polyclonal lymphocytes in which a genetic event may occur. Antigen involvement is considered to be crucial in the development of an abnormal clone of cells. The expanded clone of lymphoid cells is thought to be prerequisite for the development of monoclonal gammopathies and several infectious organisms are already associated with lymphoproliferative disorders: Ebstein-Barr virus with Burkitt's lymphoma, hepatitis C virus with low-grade non-Hodgkin lymphomas (21,22). The above mechanisms may be the evolutionary process whereby hyperreactive malarial splenomegaly transforms to splenic lymphoma with villous lymphocytes.

Acknowledgment

This work was supported by the Wellcome Trust; The Leukemia Research Fund, London; and Zeneca (formerly I.C.I.), Macclesfield Cheshire, UK.

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Received: December 17, 1997
Accepted: January 19, 1998

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