SPLENIC LYMPHOMA WITH VILLOUS LYMPHOCYTES (SLVL)

IN GHANA

Imelda Bates
BSc, MB BS, MRCP

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Department of Medicine
Komfo Anokye Teaching Hospital
Kumasi, Ghana
West Africa

Division of Haematology
St George's Hospital Medical School
London, UK

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Abstract

This thesis describes a B-cell lymphoma, splenic lymphoma with villous lymphocytes (SLVL), in Ghana. It is the first description of this disorder from tropical Africa where it may be more common than in temperate countries. In 10 patients, who could clearly be diagnosed as SLVL, the disorder is characterised by splenomegaly, lymphocytosis and typical villous lymphocytes with polar villi. In these patients evidence presented for the monoclonal nature of the lymphocytosis includes the presence of serum paraproteins, restricted immunoglobulin light chain expression and clonal rearrangement of immunoglobulin genes. The disorder differs from SLVL in Europe by affecting a younger age group and more women than men. It seems to be more aggressive than in Caucasians with a mortality rate of over 30% in 4 years.

A second group of patients is identified by this study who have some of the features of SLVL, including splenomegaly and villous lymphocytosis, but in whom no monoclonal population of cells can be detected. Some of these patients also fulfill existing criteria for the diagnosis of hyper-reactive malarial splenomegaly (HMS). The frequency with which SLVL is associated with HMS suggests that HMS may be involved in the mechanism of lymphomagenesis.

Malaria induces a polyclonal expansion of B-lymphocytes
thereby providing a pool of cells with an increased potential for mutational chromosomal events. Full tumourgenesis may also require activation of oncogenes such as BCL-1.
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CHAPTER 1

INTRODUCTION

This thesis describes a type of lymphoma in tropical West Africa characterised by specific lymphocytes in the peripheral blood and splenomegaly. The clinical, serological, immunological and molecular features are compared to those occurring in the disorder elsewhere. Reasons for its association with hyper-reactive malarial splenomegaly are suggested on the basis of analogy to the pathogenesis of other malignant lymphoproliferative disorders.

NON-HODGKIN'S, NON-BURKITT'S LYMPHOMAS IN AFRICA

Since investigation into lymphomas in tropical Africa has primarily been directed towards Burkitt's lymphoma there is limited information on other types of lymphoproliferative disorders. Non-Burkitt's, non-Hodgkin's lymphomas (NBNH) are more common than Burkitt's and Hodgkin's lymphomas and their incidence in those aged less than 60 years is higher than in temperate regions. They occur at all ages, most commonly between 20 and 50 years, and the male: female ratio is 2:1 [Fleming 1985]. It is difficult to be precise about the incidence and epidemiology of the various sub-types of lymphoma as different classification systems have been used. The majority of data concerning African NBNH lymphomas comes from Uganda's Kampala Cancer Registry with a little from
Nigeria and Zimbabwe.

Comparison with non-Hodgkin's lymphomas in temperate areas

Low-grade lymphomas only account for 17% of all NBNH lymphomas in Africa whereas in Western countries these form the majority of non-Hodgkin's lymphomas (NHL) [Levy 1988]. Africa also has a higher rate of high grade non-Hodgkin's lymphomas the histology of which has been described in publications as 'histiocytic' and 'diffuse' with a virtual absence of 'follicular' or 'nodular' sub-types [Fleming 1988, Olweny 1981]. It has been suggested that this may partly be due to delay before diagnosis [Olweny 1981] as some nodular lymphomas progress to the diffuse type before death. The presentation of NBNH lymphomas is also different; African patients tend to present later than Caucasians and have widespread, advanced disease. Many reasons for these variations have been put forward. Some geographical features of leukaemias and lymphomas in tropical Africa are similar to those of Burkitt's lymphoma which is associated with viral aetiology. It has therefore been suggested that Epstein Barr virus in association with malaria may participate in the oncogenesis of other tropical lymphoid neoplasms [Fleming 1988].

Chronic lymphomas in Africa are usually associated with splenic enlargement but they have not been widely sub-typed according to histological appearance. Lymphomas occurring in other parts of the world which have splenomegaly as a
prominent feature include 'splenic' chronic lymphocytic leukaemia (CLL), hairy cell leukaemia (HCL) and Dacie's syndrome. The most recently described entity in this group of disorders is splenic lymphoma with villous lymphocytes (SLVL).

LYMPHOPROLIFERATIONS CHARACTERISED BY PROMINENT SPLENOMEGALY

Although the spleen is involved in 34-40% of patients with non-Hodgkin's lymphoma, splenomegaly is only an isolated finding in 1-2% [Spiriano et al 1986]. Most lymphoid malignancies in which splenomegaly features prominently have characteristics which allow them to be differentiated from each other.

Hairy Cell Leukaemia

Hairy Cell Leukaemia occurs most commonly in elderly men and is usually associated with leucopenia and monocytopoenia [Bourroulec 1979]. There are no circulating plasmacytic cells and a specific pattern of bone marrow involvement is observed on trephine biopsy. The characteristic lymphocytes have circumferential hair-like projections and basophilic cytoplasm with a clear perinuclear zone corresponding to the Golgi apparatus. The cells react with monoclonal antibodies against CD25 and CD11c determinants and, in addition to B cell markers, they exhibit tartrate-resistant acid phosphatase activity.
'Splenic' Chronic Lymphocytic Leukaemia

In this variant of CLL splenomegaly and not lymphadenopathy is the predominant physical finding. Splenic CLL has been defined as a peripheral blood lymphocyte count over $15 \times 10^9/l$ and over 40% malignant cells in the bone marrow or similar numbers of bone marrow lymphocytes with a peripheral blood lymphocyte count over $4 \times 10^9/l$ sustained for at least 6 months [Dighiero et al 1979]. In CLL there is generally a higher white blood cell count than in other lymphoid malignancies and there is diffuse or nodular bone marrow involvement. The cells do not have cytoplasmic projections and are, on average, slightly smaller than normal lymphocytes. The cells possess B-cell markers and surface immunoglobulin expression is characteristically weak. The lymphocytes react with antibodies to the surface marker CD5 which distinguishes them from other lymphoid malignancies. The splenic form of CLL is unusual comprising about 5% of all cases of CLL in Europeans [Dighiero et al 1979]. However, it is reported to be the most common variety of CLL in tropical Africa [Essien 1976].

Chronic lymphocytic leukaemia in Africa

CLL is said to constitute 50-75% of all chronic leukaemias in Nigeria [Essien 1976], Ghana and Tanzania [Haddock 1967] and to be more common in West than East Africa [O'Conor 1984]. Not only does CLL in Africa differ from CLL in more developed
countries but there are also variations between West and East Africa. In developed countries a diagnosis of CLL can be made with white cell counts of only $5 \times 10^9/\text{l}$ if surface marker studies are suggestive [Bennett et al 1989]. There has been very little data published on the phenotype of CLL lymphocytes in Africa and in practise a diagnosis of CLL is made on the clinical findings of splenomegaly and a high white count which, although not defined, usually exceeds $10 \times 10^9/\text{l}$.

Half of the African patients are aged less than 50 years and in this group women predominate (M:F 1:2) [Fleming 1985, Williams 1984, Essien 1976, Allan & Watson-Williams 1963, Owor 1984] (figure 1).

<table>
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<th>Age (years)</th>
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<th>Females</th>
<th>M:F ratio</th>
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<td>&lt;44</td>
<td>16</td>
<td>27</td>
<td>0.59:1</td>
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<tr>
<td>&gt;44</td>
<td>27</td>
<td>15</td>
<td>1.80:1</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>42</td>
<td>1.02:1</td>
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[Fleming and Peters 1982]

These young patients with CLL are of lower socioeconomic status than their chronic myeloid leukaemia counterparts suggesting that factors such as malnutrition, infections and poor sanitation may play a role in leukaemogenesis [Williams 1984]. Although younger patients are also affected in East
Africa, males predominate at all ages [Fleming 1986].

The commonest presentation of African CLL is with splenomegaly rather than lymphadenopathy as in temperate zones [Williams 1984, Olweny 1981, Allan and Watson-Williams 1968, Haddock 1967] and anaemia is usual. Symptoms are generally non-specific or associated with the presence of a splenic mass. Although some patients can live for many years with few symptoms the disease tends to present late and have a poor prognosis [Essien 1976].

**Non-tropical idiopathic splenomegaly (Dacie's syndrome)**

The main features of Dacie's syndrome are massive splenomegaly with hypersplenism. There are no distinctive histological features. Retrospective analysis of histological specimens has shown that initially some patients did have a cytological abnormality in their spleens. 20% of all patients developed malignant non-Hodgkin's lymphomas of varying sub-types after 8-80 months [Dacie et al 1978]. Because of the similarities in the histological findings to Felty's syndrome and tropical splenomegaly syndrome, it was suggested that the syndrome may be an exaggerated immune response to an unknown stimulus.

The most recently described member of the family of splenic lymphomas, splenic lymphoma with villous lymphocytes (SLVL), was first described by Nieman et al in 1976.
Splenectomy Lymphoma with Villous Lymphocytes (SLVL) is a malignant disease of B-lymphocytes which has been defined in European patients. It is at least 10 fold less common than Chronic Lymphocytic Leukaemia (CLL) [Mulligan et al 1991] and has been included in the most recent classification system as a specific subtype of non-Hodgkin's lymphoma [Bennett et al 1989] involving B cells at an intermediate to late stage of maturation [Valensi et al 1990].

**Characteristics of SLVL in Europe**

SLVL can be recognised by the presence of splenomegaly and typical 'villous' lymphocytes in the peripheral blood which comprise at least 30% of the total lymphocyte count [Melo et al 1987]. It affects men more often than women (2:1) and is predominantly a disease of those over 60 years. Although in 25% of cases SLVL is detected incidentally, it usually presents with mild symptoms relating to splenomegaly or with generalised weakness and fatigue. Over 80-100% of individuals have splenomegaly with no lymphadenopathy and half have hepatomegaly. The majority of patients are anaemic as a result of haemodilution [Sp riano et al 1986]. The total white blood count is generally less at diagnosis than that in CLL (2-40 x 10^9/l) [Sp riano et al 1986, Melo et al 1987] and about 15% of SLVL patients have a white blood count less than 10 x 10^9/l.
A small percentage of cells may have plasmacytic features. 25-50% of individuals have a monoclonal band in the serum [Valensi et al 1990, Melo et al 1987] which is usually typed as IgM and a further 10-20% have a urinary paraprotein in addition or as an isolated finding. The spleen shows white pulp involvement and in 50% the bone marrow is infiltrated in a nodular or diffuse pattern.

The typical villous cells are about 7um in diameter, slightly larger than the abnormal lymphocytes of CLL. They have a round or oval nucleus, with clumped chromatin and often a nucleolus. There is a variable amount of basophilic cytoplasm. Thin, short projections, or villi, are clustered at one or both poles of the cell. Although the cells do not exhibit any specific surface antigens they are positive with antibodies for B cell markers such as CD19 and demonstrate light chain restriction. Surface expression of immunoglobulin is high compared to that in CLL and, although there are exceptions [Valensi et al 1990], SLVL cells are generally negative with antibodies for CD5. As SLVL cannot be distinguished from other lymphoproliferative disorders by surface marker studies alone, these must be used in conjunction with other cytologic and histologic parameters.

Course and Management of SLVL in Europe

SLVL seems to be a stable or slowly progressive disease with some patients remaining well for many years without treatment.
Mulligan and coworkers [1991] have followed 50 patients with SLVL for 6 months to 15 years (mean 3.7 years) 14 of whom remained well without any treatment. The most common problems experienced by patients were related to hypersplenism rather than infections or auto-immune phenomena as would be expected in CLL. 4 patients died from causes unrelated to SLVL and the rest (32) were treated with combinations of chemotherapy, splenic irradiation and splenectomy. The overall mortality of the disorder was 24%, women having a better prognosis than men. One third of all deaths were due to disease progression, half were due to vascular disease and other malignancies and in the rest the causes were unknown. The median survival was thought likely to exceed 5 years. Indications for treatment were hypersplenism, splenic discomfort or an increasing white cell count. Splenectomy was shown clearly to be the treatment of choice. 40% of patients eventually underwent the procedure and all then had white cell counts of \(3.5-5.5 \times 10^9/\)l and villous lymphocytes <10% maintained for up to 6 years without further intervention. The beneficial effects of splenectomy exceeded those that would be expected from correction of hypersplenism alone. The efficacy of splenectomy has been confirmed by other workers who found that it was followed by an improvement in symptoms, haemoglobin and paraprotein levels [Spriano et al 1986]. Other treatment modalities such as splenic irradiation and chlorambucil may be useful in those patients for whom surgery is inappropriate [Valensi et al 1990].
Information has also been published concerning the management and prognosis of other disorders similar to SLVL. Splenic CLL has a good prognosis without treatment no deaths being recorded in 23 patients in 1.5-30 years [Dighiero et al 1979]. Early splenic lymphoma restricted to the spleen and hilar nodes has a favourable prognosis after splenectomy which is thought to be 'potentially curative' [Brox et al 1991]. Splenectomy was also beneficial in splenic lymphoma in which the peripheral blood appearances were not described [Kehoe and Straw 1988].

NON-MALIGNANT CAUSES OF SPLENOMEGALY IN AFRICA

Splenomegaly is much more common in tropical areas where malaria is holoendemic than in temperate regions. In Nigeria, West Africa, the average spleen size is 271g, 100g more than in Europeans [David-West 1982]. As black Americans have spleens of comparable size to local white Americans the geographical variation may reflect a difference in environmental rather than genetic factors [Sagoe 1987].

In tropical Africa, marked splenomegaly occurs not only in lymphoreticular malignancies but also in a number of tropical infections and in the non-infectious disorder, hyper-reactive malarial splenomegaly (HMS).
Infections

Acute malaria may be associated with transient splenic enlargement which regresses with anti-malarial therapy. Malaria parasites can be found in the blood (unusual in HMS) and the spleen quickly becomes impalpable over days rather than months as in HMS. Schistosomiasis occurs in certain areas of Ghana and can be diagnosed by examination of the stool, urine or rectal biopsy for schistosomal ova. Ascites is more common in schistosomiasis than in HMS. Leishmaniasis is unusual in Ghana and is more often associated with haemorrhagic complications than HMS. It can be diagnosed in 60-80% of cases by bone marrow aspiration and biopsy [Hewlett and Pitchumoni 1987]. Other infectious causes of splenomegaly such as tuberculosis, brucellosis and typhoid fever should also be considered in the differential diagnosis.

Hyper-reactive malarial splenomegaly

HMS - formerly known as Tropical Splenomegaly Syndrome - [Bryceson et al 1983] is thought to be an abnormal response to malaria infection which occurs primarily in women of childbearing age. It is often associated with a high IgM level, which is considered to be an important criterion for its diagnosis, and hepatic sinusoidal lymphocytosis. Anaemia due to splenic pooling, increased destruction of red cells and expanded plasma volume [Pryor 1967] is a constant feature and both the splenomegaly and anaemia respond to anti-malarial
drugs. Despite fluctuations in symptoms and anaemia HMS is progressive with no spontaneous remissions and a mortality rate of 63% in 18 years [Crane 1986a]

HMS is limited to the 'malaria belt', particularly Africa and Oceania, but the incidence is low in high altitude regions. Although in the past most reports of HMS have come from the African continent and Papua New Guinea, since the publication of criteria for the diagnosis of HMS in 1981, cases have also been reported from other countries including Brazil [Alecrim et al 1982], India [Gupta et al 1987] and Indonesia [Campbell et al 1986] (figure 2).
Figure 2. Geographical distribution of reports of HMS since 1968
Ambiguity concerning the requirements for the diagnosis of HMS must raise doubts as to the validity of diagnoses of HMS prior to 1981.

The diagnostic criteria for HMS were based on work done in Nigeria and are listed below [Fakunle 1981].

**Major criteria**

- **Gross splenomegaly**: Spleen extending more than 10cm below the costal margin
- **Immunity to malaria**: Acquired by being a long-term resident in a malarious area
- **Hyerelevation of serum IgM levels**: To at least 2 standard deviations above the local normal mean
- **Response to antimalarial treatment**: With clinical, haematological and immunological improvement

**Minor criteria**

- **Hepatic sinusoidal lymphocytosis**: Occurs in over 80% of HMS patients.
- **Normal response to phytohaemagglutinin**: Wide variation but useful in differentiating HMS from CLL and lymphoma
- **Hypersplenism**: Inconsistent finding
- **Lymphocyte proliferation**: In peripheral blood and bone marrow in some African patients only
- **Familial predisposition**: Partial protection with haemoglobin AS haplotype and association with certain HLA haplotypes

The publication of these criteria was especially necessary in Africa where a myriad of conditions such as schistosomiasis,
trypanosomiasis, leishmaniasis, tuberculosis, typhoid, cirrhosis and lymphoreticular malignancies are associated with significant splenomegaly. However, these criteria are often not applied because district hospitals and rural health posts (where most of these patients are first seen) do not have access to specialist histopathological services or immunoglobulin estimations. Liver biopsy in patients who may be thrombocytopenic is hazardous in areas where supplies of emergency transfusion blood are precarious. Furthermore, several studies have shown that neither hepatic sinusoidal lymphocytosis [Bryceson 1976, Schnitzer 1976] nor high IgM levels [DeCock 1986] are specific for HMS.

8-70% of African patients with HMS have a blood and bone marrow lymphocytosis [Lowenthal et al 1980, Bryceson et al 1976]; this phenomenon has not been found in other parts of the world. An association between HMS and CLL has been suggested following the observation that a small number of people with HMS have developed CLL [Fakunle 1981]. Sagoe [1970] first described the use of lymphocyte responsiveness to phytohaemagglutinin (PHA) to assist in the differentiation between HMS and CLL in the African situation. This technique has subsequently been used in other Nigerian studies to distinguish benign from malignant causes of splenomegaly [Ukaejiofo and David-West 1979, Bryceson et al 1976]. In this technique lymphocytes were cultured in the presence and absence of PHA. Radiolabelled thymidine was added several hours before harvest and blastic transformation assessed by
either beta emission or percentage counts of lymphoblasts. In HMS, lymphocyte response was normal with over 60% of cells responding to PHA; in CLL, blastic transformation occurred in less than 40% of lymphocytes.

WORK LEADING UP TO THIS STUDY

Between 1986 and 1989 during a clinical appointment at the School of Medical Sciences in Kumasi, I conducted a study of splenomegaly in this malarious area of Ghana. During the course of the work it became clear that after locally prevalent infectious causes of splenomegaly had been excluded the remaining patients still demonstrated a heterogeneous group of disorders. Although some patients had HMS there were many cases which could not be resolved as they did not fulfill conventional criteria for either HMS or CLL. In particular, some patients exhibited behaviour typical of a lymphoproliferative disorder and had lymphocyte morphology which resembled that of SLVL. A series of serological and immunological investigations were performed in these patients to establish the identity of this disorder. The fact that DNA is a robust substance which can be collected and stored without significant degradation under tropical conditions, provided the opportunity to also carry out investigations of lymphocyte clonality and thereby to differentiate between this malignant disorder and other benign conditions such as HMS.
APPLICATION OF MOLECULAR BIOLOGY TO B-CELL DISORDERS

The techniques of molecular biology involve the analysis, or manipulation, of DNA, RNA or protein at a molecular level. In the context of the investigation of B-cell malignancies they are useful for detecting nucleotide sequence changes, such as point mutations, deletions and rearrangements, detecting clonality and determining the maturational stage of malignant cells [Norwood 1990]. During differentiation of a stem cell into a mature lymphocyte, immunoglobulin genes are rearranged. In the normal situation the B-cell population is polyclonal so many different rearrangements are present. If a single cell becomes malignant all the progeny of that cell will possess the same pattern of immunoglobulin gene rearrangement which is amenable to detection by molecular methods.

The immunoglobulin gene

Studies of immunoglobulin gene rearrangements can be used to confirm B-cell lineage and monoclonality in lymphoproliferative neoplasms such as SLVL.

Immunoglobulin molecule and gene structure

The immunoglobulin molecule consists of a pair of 2 identical heavy chains (IgH) and two identical light chains (IgL) (figure 3).
Two light chains (shaded) and two heavy chains are held together by disulphide bonds. The light chains and the heavy chains each contain one variable unit ($V_l$ or $V_h$). The light chains also contain one constant unit ($C_l$); the heavy chain constant portion has four domains ($C_h^1, C_h^2, C_h^3$ and the hinge region)
The genes encoding these polypeptides are arranged in germline configuration as discontinuous segments of DNA which contain coding segments called variable (V), diversity (D), joining (J) and constant (C). Some segments may contain hundreds of different V regions. IgH, Ig kappa and Ig lambda are located on separate autosomal chromosomes, 14q32, 2p12 and 22q11 respectively [Griesser 1989]. The heavy chain gene is the most complex [Boehm and Rabbitts 1989].

**Immunoglobulin gene rearrangements**

During the process of normal B cell differentiation segments of the immunoglobulin gene are rearranged in an orderly fashion to produce the final active form of the gene which will lead to the production of mRNA. In the production of an immunoglobulin heavy chain, first the D and J segments join (partial rearrangement) and then the DJ unites with a V segment (complete rearrangement) to form a variable region gene assembly. This, together with an intron and a constant region gene segment forms a complete rearranged immunoglobulin heavy chain gene. This unit is transcribed and spliced to constitute a functional mRNA results in the synthesis of cytoplasmic heavy chain. The synthesis of light chains is similar except that they do not possess D regions (figure 4).
Figure 4. Normal rearrangement of the immunoglobulin gene locus to form a functional kappa light chain coding gene [from Watson 1991]
The normal sequence of events in which the genes encoding the components of the antibody molecule are rearranged is heavy chain, kappa then lambda light chains. If rearrangement of the heavy chain gene is successful, attempts will be made to rearrange the kappa than lambda light chain genes. Once an immunoglobulin chain gene has been rearranged the gene on the other allele be excluded [Boehm and Rabbitts 1989]. However, if all these attempts fail the immunoglobulin protein will not be formed and the cell will remain at the pre-B stage. These events enable the cell to generate a vast repertoire of antigen receptors (ie. immunoglobulins) with unique specificities. Other mechanisms for generating diverse antigen specificity include sequence variability at the junction between V, D and J gene segments with potential for inserting 1-24 nucleotides at the ends of the junction of the immunoglobulin gene segment. This is inserted by terminal deoxynucleotidyl transferase. Somatic mutation may also occur in the genes encoding the variable region of the immunoglobulin polypeptides which by differential selection will result in antibodies of higher antigen affinity.

**Use of immunoglobulin gene rearrangement studies**

Each B cell produces an immunoglobulin with two variable region sequences of unique specificity. The progeny of these cells will be identical to the parent cell (apart from possible variation resulting from somatic hyper-mutation) and produce either kappa or lambda light chains but not both. A clonal population of cells can therefore be recognised by the
presence of a single type of light chain (light chain restriction) rather than both kappa and lambda chains. These can be detected using labelled monoclonal antibodies against the light chains. Clonality can be confirmed by analysis of the DNA rearrangement pattern of the immunoglobulin gene. In a tumour most cells have the same rearrangement because they are derived from a single cell and so specific rearranged bands can be seen on Southern blotting [Worwood and Wagstaff 1990]. This indicates that the malignancy has arisen after the B cell reached the stage of rearranging its immunoglobulin genes. Polyclonal cell populations do not show any distinct rearranged bands in Southern blotting because many different rearrangements are present. Molecular probing for the immunoglobulin gene has therefore facilitated investigation of the lineage of B cell malignancies and allowed differentiation between mono- and polyclonal lymphoid proliferations.

The relationship between monoclonality and malignancy

Generally the detection of a monoclonal population of cells implies malignancy and indicates the presence of a tumour. However there are a few instances of non-malignant disorders in which monoclonal cells can be demonstrated. Although they are associated with a high incidence of malignant transformation spontaneous regression may occur. These include T8 lymphocytosis (gamma and beta T cell receptors), varioliformis acuta, systemic Castleman's disease (a form of
reactive lymphoid hyperplasia characterised by angiofollicular lymph node hyperplasia) and lymphoepithelial lesions associated with Sjoegren's disease [Griesser et al 1989]. Lymphomas in immunosuppressed patients have oligo- or monoclonal cells whose pattern of rearrangement varies with time or site of biopsy. It is likely that during the process of lymphomagenesis oligoclonal lymphoproliferations, probably virally induced, emerge on the background of immunodeficiency. Eventually one of these clones predominates and a clonal rearrangement becomes detectable. Other events, such as chromosomal translocations involving regulatory genes, probably then occur in order to produce malignant transformation.

MALARIA AND LYMPHOPROLIFERATIVE MALIGNANCIES

Malaria prevalence and immunity
About 200 million people carry malaria infection and there is currently a resurgence due to the combined effect of resistance to insecticide and resistance to anti-malarial drugs. By the age of 1 year almost every child in tropical Africa has been infected with malaria and at least 1 million children die of the disease every year [Facer and Playfair 1989]. The life cycle of the parasite is complex (figure 5) with the anopheline mosquito as a vector. It is the schizonts that cause the intermittent fever and other symptoms of the infection. After intrahepatic maturation, merozoites are released into the blood where they invade
susceptible red cells, less than 10% of which are parasitised in *P falciparum* infections in tropical Africa.

**Figure 5. Life cycle of *Plasmodium falciparum***

SZ = sporozoite  
MZ = merozoite  
GT = gametocyte

[from Facer and Playfair 1989]
There are two different epidemiological patterns of malaria transmission. The stable form (holo- or hyperendemic) occurs where transmission is stable and frequent as in Ghana. It is characterised by repeated new infections during the course of each year. Malaria-induced immunosuppression has been demonstrated in Nigeria during trials with bacterial vaccines. Antibody responses were improved by pre-treatment with antimalarials [Facer and Playfair 1989]. Infected adults usually have very low levels of parasites and rarely present with severe symptoms of clinical malaria. In contrast, on the Indian sub-continent transmission is seasonal so malaria immunity is low and whenever infection occurs it is severe and affects all age groups. The acquisition of immunity to malaria takes about 10 years for its consolidation and is species-, strain- and stage specific. Infants are afforded some protection for the first 6 months of life by maternal, transplacentally-aquired IgG. The highest incidence of malaria occurs at 2-3 years and maximum, though not complete, immunity is acquired by 9-11 years. The reduction in malaria severity with age is associated with increasing production of malaria-specific IgG, IgA and IgM [Facer and Playfair 1989].

Association with Burkitt's lymphoma

There is now considerable evidence linking malaria with the aetiology of endemic Burkitt's lymphoma [Burkitt 1958]. Both occur within the same geographical areas where P falciparum, but not other malaria species, is hyperendemic. Variations in Burkitt's lymphoma incidence within one endemic area correlate
with factors which influence malaria transmission or immunity such as the use of insecticides, altitude or anti-malaria prophylaxis. Mice experimentally infected with malaria have a higher incidence of virus-induced lymphoma. For example, mice infected first with a murine malaria parasite and then with an oncogenic virus had a greatly increased early incidence of malignant lymphoma [Wedderburn 1970]. The malaria-related pathology was also enhanced showing that the combination of an oncogenic virus and a malaria parasite acted synergistically on the effects of both. Although the precise nature of the interaction between malaria and oncogenic viruses is not known a combination of the two factors does have demonstrable effects on the immune system. Even patients with non-clinical malaria show impaired cytotoxic T cell control of Epstein Barr virus. Lymphocytes from patients with acute clinical malaria may spontaneously transform when placed in culture. Malaria antigens can enhance the lymphocyte transformation by Epstein Barr virus and are mitogenic for human B lymphocytes.

The risk of all genetic accidents is directly related to the number of cell divisions and malaria therefore enhances the chance of a chromosomal translocation. Although the information presented here relates to Epstein Barr virus, in theory other oncogenic viruses present in the same geographical area may also interact with malaria in a similar way thereby contributing to the neoplastic process. However, in vitro it has not yet been possible to stimulate B cells carrying Epstein Barr virus with malaria antigens and produce
a cell with a chromosomal translocation. Other factors, nutritional, genetic or environmental must be involved, perhaps in the final stages of tumourigenesis which would therefore be independent of either viral or malarial infection.

PROTO-ONCOGENES IN LYMPHOPROLIFERATIVE DISORDERS

Oncogenes were discovered during experiments with acute-transforming retroviruses. Genes homologous to these oncogenes have been highly conserved throughout evolution and occur in the genomes of most vertebrate species. They appear to be part of a cell's normal genetic make-up and although in most cases their exact function is not well known they are believed to have a role in cell growth and differentiation. Epidemiological analysis suggests that several independent mutations are needed to transform a normal cell. In the experimental situation it has been shown that at least two oncogenes (such as an immortalising gene, which enables cells to grow indefinitely, and a transforming gene, which changes cell behaviour) are required to produce full tumourigenic transformation in normal cells. Oncogenes may be activated by a variety of processes including point mutations, gene amplification and overexpression and chromosomal translocation.
Endemic Burkitt's lymphoma is a common neoplasm in tropical Africa, including Ghana, in which the cells have specific chromosomal changes. These changes also occur in small non-cleaved cell NHLs and involve translocations between one of the chromosomes carrying genes coding for the immunoglobulin heavy chain (chromosome 14), kappa (chromosome 2) or lambda (chromosome 22) chains and chromosome 8 on which the C-MYC proto-oncogene is located. The pattern of breakpoint locations on chromosome 8 differs in endemic Burkitt's lymphoma in equatorial Africa compared with sporadic Burkitt's elsewhere. In the sporadic form with t(14:18) a chromosomal breakpoint far upstream of C-MYC is uncommon but is the most frequent breakpoint in the endemic type. In sporadic Burkitt's lymphoma the breakpoint frequently involves the separation of C-MYC from its regulatory region whereas in endemic lymphoma the C-MYC gene is intact except for occasional point mutations [Ambinder and Griffin 1991]. The immunoglobulin genes in B cells undergo a series of rearrangements and the C-MYC proto-oncogene is transposed into the immunoglobulin gene cluster.

Expression of C-MYC is deregulated in Burkitt's lymphoma and it is expressed at levels similar to those in normal proliferating cells [Haluska 1987]. The differing breakpoints in sporadic and endemic Burkitt's lymphoma may indicate different mechanisms for C-MYC deregulation. In endemic lymphoma the regulatory region of C-MYC remains intact and the
translocation positions C-MYC under the influence of immunoglobulin gene controlling mechanisms. In sporadic tumours the regulatory element is separated from its oncogene and transcription is initiated at sites within the first intron. The product of the C-MYC gene is involved in the regulation of the movement of cells from the resting phase into active DNA synthesis.

There is strong evidence that infection with Epstein-Barr virus (EBV) plays a major role in causing Burkitt's lymphoma and that in tropical areas, malaria is also implicated in the aetiology. Shiramizu et al [1991] have shown that 95% of tumours with breakpoints outside C-MYC were Epstein Barr virus positive whereas those with breakpoints immediately 5' of C-MYC were invariably Epstein Barr virus negative. This data suggests that Epstein Barr virus may interact with specific, but not all, types of C-MYC damage to contribute to tumourigenesis. EBV leads to polyclonal lymphoid expansion by providing antigenic stimulation and/or by direct integration into the genome of the B lymphocytes although its site of integration is not the site of the chromosomal translocations [Cheah et al 1986]. Chronic malaria infections in regions of the world where Burkitt's lymphoma is endemic may also lead to lymphoid expansion. The translocation involving C-MYC therefore occur in an abnormally expanded, but polyclonal, population of B-cells.
Oncogenes in B-lymphoid malignancies

Several lymphoid neoplasms are characterised by consistent chromosomal rearrangements. One of the partners in these reciprocal translocations is commonly an immunoglobulin gene locus (B cell malignancies) or a locus for one of the T antigen receptors chain gene (T cell malignancies). Gene rearrangements within these loci are a normal feature of lymphocyte differentiation and provide a means of producing immunological diversity. Switch sequences and heptamer-nonamer sequences involved in normal gene rearrangement processes have homology to sequences on other chromosomes making it likely that these loci are predisposed to interchromosomal as well as intrachromosomal rearrangements. The C-MYC proto-oncogene is activated in Burkitt's lymphoma; different proto-oncogenes such as BCL-1, BCL-2 and BCL-3 may be involved in other B cell malignancies. Their juxtaposition close to the immunoglobulin heavy chain gene, and less frequently the immunoglobulin light chain genes, lead to the hypothesis that they may be proto-oncogenes.

BCL-1

The BCL-1 locus is on chromosome 11 and over 63kb long. It often breaks in a region called the major translocation cluster relocating to the immunoglobulin gene heavy chain locus on chromosome 14 (figure 6).
Figure 6. BCL-1 locus

Breakpoint sites in the BCL-1 locus in lymphoproliferative disorders

CL = centrocytic lymphoma (64% rearranged)
B-PLL = B prolymphocytic leukaemia
MM = multiple myeloma
MTC = major translocation cluster
BCL-1 locus rearrangements are found in approximately 4% of B-cell neoplasms overall [Athan et al 1991]. The translocation t(11;14)q(13;q32) was originally reported by van den Berghe in 1979 and is now known to involve the BCL-1 locus and the immunoglobulin heavy chain gene. In 1984 Tsujimoto cloned the chromosome 11 breakpoint in 2 cases of B-CLL and a NHL (large cell lymphoma) cell line, which, in retrospect, were probably all cases of centrocytic lymphoma [Raffeld and Jaffe 1991]. The breakpoint site was named BCL-1 - (B cell lymphoma/leukaemia 1) - and thought to be analogous to other putative oncogenes involved in haemopoietic neoplasms. The breakpoints on chromosome 14 were within the immunoglobulin heavy chain joining region. Some studies have found that only a minority of rearrangements of the BCL-1 locus involve the immunoglobulin heavy chain joining region [Rimokh et al 1990, Midieros et al 1987]. This suggests that either the rearrangements were not the result of a t(11:14) translocation or that the break on chromosome 14 occurred outside the J gene region. Although there were sporadic reports of these abnormalities occurring in lymphocytic leukaemias and lymphomas and additional breakpoints were identified, no messenger RNA gene product from this site could be detected.

BCL-1; association with centrocytic lymphoma

There is recent evidence linking the t(11;14) translocation to a distinct lymphoma subtype, centrocytic lymphoma [Williams et al 1991]. Although once thought to be distinct, it is now
clear that this is synonymous with a lymphoma described in American literature as lymphocytic lymphoma of intermediate differentiation. Centrocytic lymphoma only accounts for 5-10% of all non-Hodgkin's lymphomas. It is derived from follicular mantle zone cells and defined in the Kiel classification as being composed of small, cleaved lymphocytes without the presence of transformed lymphocytes. It has a diffuse or vaguely nodular growth pattern and the cells usually react with antibodies to the determinants CD19, CD20, CD22 and CD5 but not CD10. They have high density surface immunoglobulin and membrane associated alkaline phosphatase which also occurs in normal follicular mantle cells. The cells may spill over into the peripheral blood and be indistinguishable on morphological grounds from some forms of CLL. 30-55% of centrocytic lymphomas are reported to have a BCL-1 translocation but this is likely to be an underestimate because most workers only studied the major translocation region and several other breakpoint sites are now known to exist [Raffeld and Jaffe 1991].

**BCL-1 involvement in non-centrocytic lymphomas**

As centrocytic lymphoma has only recently been well defined it is difficult to be confident that some previously reported cases of lymphoproliferative neoplasia were not in fact centrocytic lymphoma. BCL-1 translocations have been found in 4-6% of small lymphocytic lymphomas, 6% of adult B cell lymphomas, 3% of multiple myelomas and 20% of prolymphocytic
leukaemias.

Cytogenetic studies in CLL are relatively recent and most information about proto-oncogenes has been gained from the study of rare cases of CLL with chromosome translocations. The most common cytogenetic abnormality is trisomy 12 followed by translocations from largely unknown donor chromosomes to 14q, 13q, and 11q. The translocations involving chromosome 14 usually break at band q32, the site of the immunoglobulin heavy chain gene, and typically the donor is chromosome 11. The t(11;14) (q13;q32) is less common in CLL than in B-prolymphocytic leukaemia. Rearrangements of the BCL-1 locus have recently been described in a few patients with SLVL some of whom did not have detectable chromosomal translocations at the BCL-1 site [D Jadayell, personal communication].

**The effect of the BCL-1 gene**

By analogy with C-MYC and BCL-2 (see later) it seemed likely that the t(11;14) translocation would affect the transcription of a postulated cell growth gene close to the BCL-1 locus located upstream of the immunoglobulin enhancer. It was known that, in general, activated oncogenes are frequently associated with Hpa II-tiny fragment (HTF) islands. Chromosome walking techniques were used to isolate genomic clones extending from the sites of translocation in a 3' direction to the first HTF island. The first gene that was found was activated in cell lines with the t(11;14)(q13;q32)
translocation and was found to code for a protein whose amino acid sequence predicted it to be member of the cyclin family [Withers et al 1991]. Alterations of cyclins, which are cell cycle regulators, might be expected to lead to altered cell cycle progression. This gene has not yet been conclusively identified but the most likely candidate is the parathyroid adenomatosis gene 1 (PRAD 1) on chromosome 11q13. In parathyroid adenomatosis this is deregulated by being rearranged with the parathormone locus on chromosome 11p15 and it has homology to cyclins. The PRAD 1 gene is often expressed in lymphoproliferative disorders with the t(11;14) translocation and is only 200kb away from the BCL-1 locus. It encodes a mRNA transcript of 4.4kb which may be detected along with other sized fragments (eg. 1.35kb) by Northern blotting. Most human cell lines, but not those derived from bone marrow stem cells, have detectable levels of this mRNA.

**BCL-2**

The t(14;18), t(2;18) and t(18;22) translocations juxtapose the immunoglobulin heavy chain gene (as in many follicular lymphomas) or the kappa or lambda light chain genes respectively with the putative proto-oncogene BCL-2. This proto-oncogene is involved in 85% of follicular centrocytic/centroblastic lymphomas. High levels of BCL-2 gene expression normally occur during pre-B cell development. As the cell matures expression is normally downregulated although BCL-2 may be re-expressed if the B-cell activated [Annotation
1990]. Translocation of BCL-2 to chromosome 14 prevents the normal downregulation. The BCL-2 gene product is a protein located on the inner mitochondrial membrane which, in certain settings, prevents apoptosis thereby prolonging cell survival. Apoptosis can be differentiated from cell necrosis by the presence of nuclear fragmentation and chromatin condensation rather than nuclear and cytoplasmic swelling. Somatic mutation in the variable region of the immunoglobulin gene is associated with production of high affinity antibodies in response to T cell dependent antigens. Proliferating follicular centre cells which do not encounter high-affinity antigen undergo apoptosis or programmed cell death. Those cells whose receptors do encounter high-affinity antigen enter the resting memory B cell pool. BCL-2 is likely to be responsible for protecting B lymphocytes with high-affinity receptors from apoptosis [Hockenberry et al 1990].

**BCL-3**

In the t(14;19) translocation the immunoglobulin heavy chain gene is joined to BCL-3 another putative proto-oncogene whose gene product is increased as a result. This product is thought to be involved in controlling the cell cycle and cell lineage differentiation.

There is a striking similarity between these rearrangements and those underlying Burkitt's lymphoma. The predilection for these rearrangements is probably related to the recombination...
events that take place within the immunoglobulin heavy and light chain loci during normal B-cell development. In the case of BCL-2 there is direct experimental evidence for its ability to act as an oncogene. Transgenic mice with a BCL-2/immunoglobulin minigene in which human BCL-2 was overexpressed in their B-cells, developed lymphadenopathy with follicular B-cell infiltrates. There was a polyclonal expansion of resting B cells with prolonged cell survival but no increase in cell cycling [McDonnell 1989].
CHAPTER 2

METHODS

LOCATION OF STUDY

Ghana is a country of similar size to the UK (area 239,000 square kilometers) with a population of 14 million. The capital city of Accra has a population of one million. This study was carried out in Kumasi which is situated in the central part of the country (figure 7) in the Ashanti region and is the second largest city with a population of 380,000. Kumasi lies within the tropical rain forest belt at a height of 400m, latitude 6° 41'N and longitude 1° 38'W. There are 'big' and 'small' rainy seasons from April to July and September to November and the annual rainfall is 1000-2100mm [Gordon 1990]. Malaria is hyperendemic in the region (ie. transmission occurs throughout the year and produces splenomegaly in over 50% of children) and is almost exclusively due to Plasmodium falciparum.
Figure 7. Map to show location of study
The health care services in the country are centred round regional state-funded hospitals administered by the Ministry of Health and primary health care centres staffed by full-time nurses with support from doctors. In addition there are many mission hospitals and health posts funded by church organisations and staffed by both expatriates and local Ghanaians. They tend to be better equipped than government hospitals because much of their finance comes from organisations in more wealthy countries. Private hospitals also exist and are utilised by the more wealthy patients. General practitioners practise in the larger towns but are not paid by the government and make their living by charging patients directly for their services.

Patients pay for all their medical care including drugs and hospital accommodation. One night's stay in a state hospital costs about half a day's wage but basic drugs prescribed within the hospital such as penicillin are free. Other drugs have to be purchased from local pharmacies at commercial rates so medicines which are not manufactured in the country are expensive. Most investigations must also be paid for - for example, a chest X ray costs about one day's wage and a prothrombin time almost two day's wages.

This study was carried out at Komfo Anokye Teaching Hospital, a 1000 bedded state-owned, tertiary referral centre, named after a local folk hero (photograph 1). It is one of two hospitals in the country with a medical school, the other
being Korle-Bu hospital in Accra. It has a catchment area covering about one million urban- and rural-dwelling people. Most patients admitted to this hospital cannot afford private health care and make their living by subsistence farming or petty trading. A haematology service was established in 1986 by myself; prior to this there had not been any specific provision for haematology patients. Initially the clinic was conducted in the general medical ward but after 1 year a microscope was provided and a small room was allocated specifically for haematology. About 300 patients a year were referred to the clinic from specialists within the hospital, local GPs or rural hospitals and health posts. The range of haematological problems encountered in the clinic included nutritional anaemias, malignant disorders, glucose-6-phosphate dehydrogenase deficiency, anaemia of pregnancy, haemoglobinopathies and undiagnosed splenomegaly.
Photograph 1. Komfo Anokye Teaching Hospital, Kumasi
SOURCE AND SELECTION OF PATIENTS WITH SPLENOMEGALY

The patients in this study were recruited from the haematology clinic at Komfo Anokye Teaching Hospital in Kumasi during the period 1986-1990. The vast majority were residents of the Ashanti region (population 4 million) although many had their origins in other parts of the country. Most non-pregnant patients (80%) were referred from the department of medicine, 6% from district hospitals and the rest from other hospital specialities and the chief laboratory technician. The obstetric department referred 82% of all pregnant women to the clinic, 15% came from the medical unit and 3% from district health posts.

As splenomegaly is such a common finding in tropical Africa this study was limited to patients with spleens measuring at least 10cm from the costal margin (see later). The few patients cited in this work with spleens of less than 10cm were included because they had been known to have massive splenomegaly which had been treated medically or surgically prior to presentation at our clinic. The most common causes of gross splenomegaly in this part of Africa are schistosomiasis, lymphoreticular malignancies and portal hypertension; it is not uncommon for two or more diseases to coexist in the same patient [Lowenthal et al 1980, Rees et al 1982]. All patients in whom the cause of splenomegaly could be shown to be due to infections, portal hypertension or non-lymphoid haematological disorders were excluded from the
study.

CATEGORISATION OF PATIENTS WITH HMS, CLL AND SLVL

Hyper-reactive malarial splenomegalY

The diagnostic criteria for HMS have been discussed previously (see introduction). All patients fulfilled the two major criteria of splenomegalY and malarial immunity as they had all been long-term residents in the area of the study which is holoendemic for malaria. IgM levels could not be measured in Ghana but serum was collected to enable this measurement to be carried out retrospectively.

All patients initially thought to have HMS were prescribed proguanil 100mg/day except pregnant women who were given chloroquine base 150mg twice a week. Although proguanil is safe and efficacious in pregnancy the obstetricians at the hospital were more familiar with chloroquine as prophylaxis and it was hoped that compliance would be better if the therapy conformed to the usual practice within their department. Follow-up of the patients' clinical condition, spleen size, haemoglobin and peripheral blood lymphocyte count was continued for as long as possible. The documentation of response to treatment was not straightforward as the improvement in spleen size and various haematological parameters was occasionally reversed by intercurrent illnesses
such as infections and haemolytic episodes. Drug compliance was also difficult to ensure and a lack of drugs for several weeks would result in cessation or reversal of splenic regression.

In common with other studies in developing countries [Ukaejiiofo et al 1987] about 50% of patients did not report back to the clinic so that although a clinical diagnosis of HMS was made at the initial presentation, this could not be confirmed by documenting a good response to anti-malarial treatment. Financial constraints were the main reason for this failure of clinic attendance. HMS requires lifelong treatment and patients are expected to buy their own drugs. In addition many people have to travel long distances to reach the hospital. There is a lack of incentive to attend clinics as most patients with HMS are not severely incapacitated. However, a definitive diagnosis of HMS cannot be made without assessing the response to treatment so it is essential to ensure adequate follow-up. The following strategies were adopted to encourage patients to attend for follow-up:

- consultations were held in a quiet room away from the main out-patient area
- free drugs were provided
- patients were seen at least once a month by the same doctors (myself or Dr Bedu-Addo) and technician (Mr Charles Ampong)
- the need for strict drug compliance was stressed and the consequent reduction in splenic size was illustrated pictorially for the patients at each visit - consultations in the same clinic were provided for the patients' immediate family free of charge

Liver biopsies to assess the lymphocytic infiltration of the sinusoids was not performed routinely as without a clotting screen and adequate emergency blood transfusion services the procedure was considered too hazardous.

**Chronic lymphocytic leukaemia**

The diagnosis of CLL is usually more straightforward than that of HMS; criteria for its diagnosis have been published by the International Workshop on Chronic Lymphocytic Leukaemia [Bennett et al 1989]. These are a sustained peripheral blood lymphocyte count >10 x 10⁹/l and bone marrow lymphocytes >30%. The lymphocytes in CLL are generally slightly smaller than normal lymphocytes. Chlorambucil was not available in Ghana at the time of the study. The few patients who managed to acquire chlorambucil from abroad were treated for a short period initially but none received any maintenance therapy.

**Splenic lymphoma with villous lymphocytes**

Patients with SLVL were selected from groups of individuals
with presumptive diagnoses of either HMS or CLL who attended the haematology clinic at Komfo Anokye Teaching Hospital, Kumasi between 1986 and 1991. They were identified by the presence of at least 30% of total circulating peripheral lymphocytes having the appearance of typical villous lymphocytes [Melo et al 1987].

Haematological, serological, immunological and molecular investigations were carried out patients from each of these diagnostic categories to identify parameters that would distinguish them from each other and to more fully describe SLVL in Ghana.

**DOCUMENTATION OF CLINICAL FEATURES**

**Symptoms**

In addition to routine questions patients were particularly asked about the following:

- their reasons for seeking medical help
- the duration of the splenomegaly
- pain or discomfort associated with the spleen
- whether the spleen had been static or enlarging.

They were also asked how often they had had 'fever' - implying malaria - and a family history of splenomegaly was sought as HMS has been reported to have a genetic basis (see
discussion).

**Signs**

At the initial consultation, clinical evidence of the following abnormalities was documented:

- fever
- anaemia
- jaundice
- lymphadenopathy
- tachycardia
- ejection systolic murmur
- ankle oedema
- hepatomegaly

**Measurement of spleen size**

At the first, and every subsequent visit, the spleen size was measured in the same way by myself or my colleague Dr. George Bedu-Addo. Great care was taken to ensure that this measurement was reproducible because alterations in splenic size were used to assess the efficacy of treatment. Our inter-observer error was not more than 2cm if the following method was used. The patient lay flat on a firm bed or on the floor with no pillows and without being rotated to either side. Their hands were placed by their side and they were asked to breathe normally without excessive inspiratory or expiratory movements. The tip of the spleen was located by gentle palpation and its position confirmed by percussion. The
distance from the left costal margin in the anterior axillary line to the tip of the spleen, following the direction of the spleen was measured with a tape measure. The anterior axillary line rather than the mid-clavicular line was chosen because in gross splenomegaly the spleen tends to enlarge sideways as well as downwards causing a bulge in the left flank. A more lateral starting point therefore allowed the contour of this sideways expansion to be taken into account. Particular care was taken in obese patients not to push the fingers, and consequently the tape measure, into the fat over the tip of the spleen. The length of the spleen was measured along the skin surface as indentation of the adipose tissue could lead to over-measurement of the spleen by 2-5cm. In addition, the overall shape and consistency of the spleen and the presence, or absence, of a splenic notch were noted and indicated on a diagram which was drawn for each patient. The size and shape of the liver and, where appropriate, the uterus were also shown (for examples see figure 8)
Figure 8. Diagrams used to illustrate abdominal findings
LABORATORY INVESTIGATIONS

Investigations carried out in Ghana

The following investigations were performed at KATH with a view to providing baseline data or excluding causes of splenic enlargement other than HMS and CLL. Investigations listed in group 1 were carried out on all patients, whereas those in group 2 were only done if specifically indicated. The cost of basic investigations had to be borne by the patients themselves and therefore could not be justified at every visit. Tests were only requested at the first consultation or if indicated later by the patients' clinical condition.

**Investigations on all patients.**

- Haemoglobin
- Total and differential white cell count
- Stool and urine microscopy for cells, ova and parasites
- Microscopy of stained blood and bone marrow aspirates
- Haemoglobin electrophoresis (cellulose acetate)

**Investigations on selected patients.**

- Reticulocyte count
- Direct Coombs' test
- Glucose-6-phosphate dehydrogenase deficiency screen (methaemoglobin reduction test)
- Thick film for malarial parasites
- Concentrated stool examination
Urine urobilinogen
Serum total and unconjugated bilirubin
Liver biopsy
  Sputum culture and microscopy
  Chest X ray
  Oesophagogastroduodenoscopy
  Scanning electron microscopy of red cells

Analysis of blood and bone marrow slides

Anti-coagulated blood collection bottles were not always available so peripheral blood slides were made from finger prick specimens. Bone marrow slides were prepared fresh from aspirated samples at the bedside. The slides used were often scratched as they had to be scrubbed clean and recycled. The blood and bone marrow smears were taken by myself or Dr. Bedu-Addo and reported by myself after being stained in the laboratory by the standard method of Leishman.

Haemoglobin electrophoresis

The electrophoresis was carried out by myself using a Gelman sepratek system. A few drops of blood were collected from a finger-prick and put into 1ml normal saline. The sample was washed three times before being resuspended in a few drops of saline and then frozen and stored for up to three months. Specimens were defrosted 1 hour before being used (thereby lysing the cells) and diluted approximately 1 in 1 with
distilled water until the lysates were all at roughly equal concentrations. The samples were applied to the wetted cellulose acetate paper using the applicator provided in the kit, and an electric field of 200V was applied for 40-45 minutes using a continuous buffer (tris-EDTA-glycine buffer pH 9.2) system. The paper was then stained in Ponceau S for 10 minutes and rinsed and decoloured in a 5% acetic acid solution until a white background was obtained. During electrophoresis the high ambient temperatures and humidity caused overheating and condensation dripped from the lid onto the paper during the procedure. The voltage was therefore reduced from the recommended 400V to 200V and the time for each run increased from 30 to 40-45 minutes. The lid was wiped several times during each procedure and between runs the buffer was allowed to cool down completely. The direction of the current was reversed for the next run. Electrophoresis was also carried out on 76 normal geographically matched controls.

Other investigations

All other tests carried out at KATH were performed by technicians in the haematology, biochemistry, microbiology and parasitology laboratories, by radiographers and by colleagues in the department of medicine.
Investigations carried out in UK

Scanning electron microscopy of red cells

Unlike CLL, SLVL does not appear to be associated with a particularly high risk of infection. The enlarged spleens must therefore be immunologically competent. The usual methods of assessing splenic function such as radionucelotide scanning or clearance rates of abnormal radiolabelled red cells were not available in Ghana. However, the technique of counting pocked (or pitted) red cells in the circulation can be employed on Ghanaian blood because appropriate samples can be successfully transported in fixative for analysis in the UK. This method is particularly appropriate for studying splenic function in chronic diseases because it gives an assessment of basal splenic activity rather than splenic function at a single moment in time. The number of poked red cells correlates well with the technetium spleen scans and both are sensitive to all but the mildest degrees of splenic dysfunction [Pearson 1985].

The pocks are autophagocytic cytoplasmic vacuoles which are removed from the cell by fixed macrophages in a normally functioning spleen. Usually less than 2% of circulating red cells have two or more pocks but this figure rises to 25-70% in splenectomised subjects [Buchanan 1987]. Intermediate numbers of pocks occur in patients with partial splenic hypofunction such as those with haemoglobin SC disease [Pearson 1979]. In sickle cell disease splenic dysfunction is
associated with severe infections with encapsulated organisms similar to the sepsis which occurs in post-splenectomy states. If the patients in this Ghanaian study have hyposplenism and a predisposition to develop infections they too would be expected to have increased numbers of circulating pocked red cells.

In order to carry out electron microscopy on samples from Ghanaian patients three drops of fresh whole blood were fixed in 0.5ml 3% glutaraldehyde in phosphate buffer and stored for up to 8 weeks before shipment to the UK. Previous work has shown that the percentage of pocked red cells does not change significantly in mixtures of blood and glutaraldehyde stored at 20°C for less than three months [Pearson 1985]. Further processing was carried out at St George's Hospital by Mr Ray Moss. The tissue was post-fixed in osmium oxide for 1-2 hours and then rinsed in buffer. Dehydration was carried out using a graded series of ethanol and this was removed by graded series of freon. Samples were transferred to closed critical point drying pots, dried and mounted onto clean aluminium stubs before being gold-coated prior to viewing.

**Immunoglobulin M levels**

At the patient's first visit to the clinic samples of serum were obtained whenever possible. Serum was separated from whole blood either by centrifugation or by leaving the samples to stand in the fridge overnight. Specimens were stored at -
20°C for up to 3 years before being processed in the UK. For transportation from Ghana to the UK the samples were packed into polystyrene containers for the parts of the journey involving travel by road (6 hours) or plane (9 hours) and it is likely that some degree of thawing took place during this time.

The immunoglobulin M assays were carried out by myself using NOR-Partigen radial immunodiffusion plates. A precipitate is formed in the gel the square of its diameter being proportional to the level of IgM in the serum. 5ul of serum from each patient, were placed into the precut wells. A control sample was also included in each plate and 3 standards were used to enable a reference curve to be constructed. The plates were left at 37°C in a humid atmosphere for 4 days to allow the IgM to diffuse into the gel and the diameters were then measured to within 0.1mm. IgM levels were read directly from the reference curve. IgM levels were also measured in 19 healthy blood donors residing in the same locality as the patients.

**Serum protein electrophoresis and immunofixation**

This procedure was carried out using the same system and buffer as haemoglobin electrophoresis. 20ul samples of serum were loaded into large size wells and applied to the cellulose acetate paper with an applicator. A current of 150V was used for 50 minutes and the protein bands stained with Ponceau S.
A control sample (pooled Ghanaian sera from healthy volunteers living in the same area as the study patients) was included in the central position in each run. The procedure was repeated using 1:3 dilutions of sera from patients with particularly high background staining in whom small paraprotein bands may have been overlooked. Samples from all patients with >30% villous lymphocytes in whom abnormal bands were detected on screening were further analysed by immunofixation to detect and characterise any monoclonal bands. This technique involved electrophoresis of 6 serum samples from each patient at dilutions of 1:50 for 50 minutes at 150V. One lane was then stained with weak Nigrosin for 5-10 minutes and washed on 0.9% saline for 10 minutes. Pieces of membrane were soaked in a 1:10 dilution of antiserum in 4% polyethylene glycol 8000 in phosphate buffered saline and applied to the appropriate lane for 15 minutes. Care was taken to exclude any air bubbles. The antisera used were against IgG, IgM, IgA and kappa and lambda light chains. After 15 minutes excess antibody was washed off in 3% saline containing 0.1% polyoxyethylene sorbitan (Tween 20) for 20 minutes. At the end of this time the membrane was stained with Nigrosin and examined with back lighting for the presence of stained monoclonal bands in the lane to which the specific antibody had been applied. This allowed identification of any multiple monoclonal bands which may have been present and typing of both heavy and light chains.
Detection of surface antigens and membrane-associated light chains

Peripheral blood smears for confirmative morphology and surface marker studies were prepared in Ghana from finger-prick or EDTA samples. They were allowed to air-dry before being wrapped individually in aluminium foil and placed in airtight boxes containing silica gel. These were stored at -20°C and transported to UK via personal or commercial courier. Despite shipment at ambient temperatures no deterioration of lymphocyte surface antigens was detected in those slides which reached the laboratory within 5 days of leaving Ghana. However it was found to be essential that the slides were kept dehydrated in silica gel. The slides were defrosted in the UK immediately prior to use and cell morphology ascertained by light microscopy after staining with Wright's stain.

For the APAAP technique, [Cordell 1984] the smears were fixed in a mixture of methanol, acetone and formaldehyde (19:19:2) for 60-80 seconds before washing with tris-buffered saline (pH 7.6). A 1cm diameter circle was marked on the slide into which 10ul of the monoclonal antibody were dropped. Antibodies against the determinants CD2, -4, -8, -19, -5 and -11c and against the light chains kappa and lambda were used. The characteristics of these CD antigens are listed below.

CD2 - : a 50kD molecule on the surface of the majority of circulating T cells which is the receptor
responsible for binding sheep red cells in the E-rosetting assay.

\textit{CD4} - a 55kD protein present on most human T cells of helper/inducer subtype which is the receptor for the HIV virus and HLA class II antigen-presenting cells

\textit{CD5} - a 67kD molecule which is present on the surface of more than 95% of normal human T cells. It is also found on the neoplastic cells in B-CLL and centrocytic lymphoma but not on normal B cells. It specifically interacts with the cell surface protein CD72 which is exclusive to B cells [Van de Velde et al 1991]

\textit{CD8} - a molecule of 33kD present on human suppressor/cytotoxic T cells and also with sinus lining cells in the human spleen

\textit{CD11c} - the 150kD alpha-chain of the p150,95 molecule present on macrophages and hairy cells. This protein belongs to a family of related heterodimeric proteins which share a common beta chain. The other members of the CD11 group are CD11a, leucocyte function associated antigen 1 and CD11b aC3bi receptor.

\textit{CD19} - a 95kD polypeptide which appears prior to the pre-B cell stage and is lost shortly before the terminal plasma cell stage. The antibody will therefore detect both normal and neoplastic B cells but not plasma cells.

The slides were incubated for 30 minutes with the monoclonal
antibody and, after washing, were incubated for a further 30 minutes with rabbit-anti-mouse antibody and normal human serum to block complement receptors. The slides were washed and incubated for 30 minutes with the APAAP complex - a mouse anti-alkaline phosphatase antibody complexed with alkaline phosphatase. The last 2 steps were repeated for 10 minutes each to amplify the final colour signal. Substrate mixture was applied and left for 30 minutes. Levamisole (1mM) was added to the substrate mix to block any endogenous alkaline phosphatase activity. The slides were then counterstained with haematoxylin, mounted and examined by normal microscopy.

A negative control was included in each experiment by omitting the first antibody layer. Positive controls from normal individuals or those with CLL or hairy cell leukaemia were included in each experiment as appropriate. Monoclonal antibodies were also applied to slides from 9 healthy Ghanaian controls (8 males: 1 female; 23-31 years, mean age 27) who did not have splenomegaly or villous lymphocytosis and resided in the same locality as the patients.

**Extraction and purification of DNA**

At the start of this study reagents for DNA extraction were not available in Ghana so whole blood was transported at -20°C to UK. As the system for reagent supply from London to Kumasi became established the technique of DNA extraction was altered to enable DNA to be obtained from whole blood in Ghana and
transported to UK as impure DNA.

The original method involved collecting 20ml samples of blood which were allowed to clot and then separated from the serum and frozen prior to transportation to UK. In the UK they were thawed quickly at 37°C, roughly chopped and homogenised manually in cell lysis buffer (0.32M sucrose, 10mM tris pH 7.5, 5mM magnesium chloride, 1% Triton X-100). The nuclei were recovered by centrifugation and digested by overnight incubation at 37°C in a buffer (75mM sodium chloride, 24mM EDTA pH 8.0) containing sodium dodecyl sulphate and proteinase K. Residual proteins were precipitated by the addition of saturated sodium chloride and the tubes spun at 2400rpm for 15 minutes. Two volumes of ethanol were added to the supernatant to precipitate DNA which was then recovered by spooling.

In the later method, 20ml EDTA anti-coagulated blood were mixed with 50ml cell lysis buffer and after centrifugation at 2500rpm for 10 minutes the nuclear pellet was suspended in 1-2ml guanidinium thiocyanate buffer (50g guanidinium thiocyanate, 0.5g sodium N-lauryl sarcosinate, 2.5ml 1M sodium citrate (pH 7), 0.7ml 2 mercapto-ethanol, water to 100mls) to lyse the nuclei and stored at ambient Ghanaian temperatures for 1-9 months until transported to the UK. The DNA was then banded in cesium chloride by ultracentrifugation (32000 rpm for 16 hours) and dialysed against 10mM tris, 1mM EDTA ph 7.5 (1 x TE) for several days to remove traces of cesium chloride. This method yielded adequate amounts DNA to perform Southern
blots (13-3245ug) the largest yields coming from leukaemic patients. The concentration of DNA was determined by absorbance at 260nm and optical density of 1 being taken as equivalent to 50ug/ml. The quality of the DNA was assessed by electrophoresing 0.5ug of DNA through a 0.8% agarose gel. In the majority of samples only large size DNA was present. In a few samples, there was in addition, a small amount of degradation as evidenced by smearing on the gel. Even in these samples there was sufficient undegraded pure DNA to allow endonuclease digestion (photograph 2) [Bates et al 1991].
Photograph 2. Comparison of quality of 5ul samples of DNA obtained by extraction from:

a - frozen blood clots

b - guanidinium thiocyanate buffer
Detection of immunoglobulin gene rearrangements

High molecular weight DNA was digested overnight with at least two of the following restriction enzymes; Hind III, Bam H1, EcoRI, BglII. The manufacturer's recommended buffers were used to which 1-2mmol spermidine were added to overcome endonuclease inhibition [Bouche 1981]. An aliquot of this DNA mixture was added to phage lambda DNA as a control and left to incubate for 1-3 hours in parallel with the main digest. The control sample was then electrophoresed through a 0.8% agarose mini-gel at 70V for 45 minutes to confirm successful digestion. The samples which had digested were ethanol precipitated and the DNA fragments size-fractionated on 0.8% agarose gels before being blotted onto Hybond N+ nylon membranes overnight using 3M sodium chloride, 0.3M sodium citrate (20 x SSC) buffer. The DNA was fixed onto the membranes by soaking in 0.4M sodium hydroxide for 20 mins and the membranes were rinsed in 2 x SSC.

The probe used for the immunoglobulin heavy chain gene was for a 2.5kb region of the joining region and was obtained from T Rabbitts, Cambridge. It was labelled with $^{32}$P by nick-translation and the incorporation and specific activity calculated on the counts per minute (as measured on a beta counter) emitted by aliquots of the original reaction mix and the final probe. Probes used had activities of $1 \times 10^7$ - $1 \times 10^8$ dpm/ug of DNA and radioactive incorporation efficiency was 30-60%. The membrane was pre-hybridised for at least 1 hour at
65°C in a buffer containing 25ml 20SSC, 5ml 100 Denhardt’s, 5ml 10% SDS and 65ml water. 500ul/membrane of boiled, sheared salmon sperm were added to prevent non-specific binding. The probe was boiled to separate the DNA strands, diluted with a little hybridisation buffer, added to the membrane and left to hybridise to the DNA on the membrane in a shaking water bath at 65°C overnight. The membrane was then washed in three increasingly stringent salt solutions (2 SSC/0.1%SDS, 1 SSC/0.1%SDS, 0.1 SSC/0.1%SDS) for a total of 1 hour and autoradiographed. An intensifying screen with two films was used, one film being developed after 2-4 days and the other after 7-10 days.

A lambda Hind III size marker was run on each gel and the distance of each size band from the loading wells measured when the ethidium bromide-stained gel was viewed under UV light prior to blotting. A graph of DNA band size against distance run on the gel was plotted and used to determine the size of any bands seen in the tracks of sample DNA. The size of the germline band expected with each enzyme is known and so the size of any abnormal bands, indicating the presence of clonal gene rearrangements, could be determined from the curve.

After completion of radiography the membranes were stripped in a boiling solution of 0.1%SDS. Complete removal of the probe was confirmed by autoradiography. The DNA on the membranes was then screened for rearrangements of the BCL-1
locus by the same method as before but using a probe for the major translocation cluster of the BCL-1 locus instead of the J" probe.

**Statistical analyses**

Whenever possible the results were subjected to statistical analysis by comparing proportions using the $x^2$ test (haemoglobin electrophoresis), obtaining $p$ values for correlation coefficients (villous lymphocytes related to spleen size, age and total lymphocyte count) or $t$ tests on groups of individual observations (all others).

**Problems encountered in setting up a new research project in the tropics**

**Clinical**

Clinical problems centered around patients' poor clinic attendance and non-compliance with therapy and have already been discussed.

**Administrative**

Prior to 1986 Ghana had only one haematology clinic which was located in Accra. Before beginning this study it was therefore necessary to establish a clinical haematology service in Kumasi and to provide a referral service for haematological patients. This involved acquiring a suitable location, a microscope, a centrifuge, bone marrow needles and a supply of
new slides and reagents. To avoid the problem of long patients queues to collect medical notes from the hospital records system patients were provided with school notebooks which they kept in their possession.

**Technical**

Although techniques for slide preparations and DNA extraction from mononuclear cells are well established in Europe, suitable reagents and equipment were not available in Ghana. Unique methods of sample collection appropriate for tropical conditions had to be devised. The efficacy of these techniques often took several months to establish due to transport difficulties between Kumasi and London and the quality of the samples was occasionally sub-optimal. All consumable items were in short supply so blood tests were done on finger-prick specimens and kept to a minimum as patients had to pay for tests themselves. Glass blood slides were scrubbed clean and reused, the scratches often making morphological interpretation difficult. For several months these problems were compounded by the use of local gin to fix the slides when supplies of methanol ran out.
CHAPTER 3

RESULTS

During routine examination of peripheral blood films from Ghanaian patients with splenomegaly the presence of villous lymphocytes was noted on several samples. Large numbers of these cells are found in the blood of patients with splenic lymphoma with villous lymphocytes (SLVL), a disorder not previously described from tropical Africa. To determine whether these Ghanaian patients did indeed have SLVL, clinical, morphological, immunophenotypic and molecular data were collected and compared to previously described cases of SLVL from western countries.

Peripheral blood films from 151 Ghanaian patients with splenomegaly were examined for the presence of typical villous lymphocytes. The cellular morphology was sufficiently well preserved in 93 of these to allow assessment of lymphocyte appearance. The peripheral blood lymphocyte count (PBLC) in these 93 patients varied from $0.3\rightarrow1,000 \times 10^9/l$ and its relationship to the percentage of villous lymphocytes is shown in figure 9. There was a significant association between the PBLC and the number of villous lymphocytes ($p < 0.01$) the latter being higher in patients with the greatest lymphocyte counts.
Figure 9. Relationship between PBLC and percentage of villous lymphocytes

PBLC = peripheral blood lymphocyte count (excluding patient with PBLC >1000)
The majority of these patients had been diagnosed as either African chronic lymphocytic leukaemia (CLL), with splenomegaly and a peripheral blood lymphocyte count (PBLC) >10 x 10⁹/l, or hyper-reactive malarial splenomegaly (HMS). A presumptive diagnosis of HMS was made at the first clinic visit on the basis of long-term residence Ghana and splenomegaly of at least 10cm for which no locally prevalent cause could be found. The diagnosis was confirmed if the patients had a good response to anti-malarial therapy (ie. at least a 40% reduction in splenic size without any recurrence on treatment). IgM levels were measured in retrospect; high levels supported a diagnosis of HMS.

APPEARANCE OF VILLOUS LYMPHOCYTES

The villous lymphocytes were 7-9um in diameter with a round or slightly oval nucleus of regular outline. A single nucleolus was commonly observed and the cytoplasm was usually more abundant at the poles of the cell and mildly or moderately basophilic. The villi could be categorised as either 'hair'-like (the most common type) which were fine projections 1-2um long, or 'blebby'. These were wider, blunter projections. Both types of villi were concentrated at one or both poles of the cell and the type of villous was constant for each patient (see photograph 3).
Photograph 3. Villous lymphocytes in Ghanaian patients by light microscopy (a, b and c) and scanning electron microscopy (d)
CATEGORISATION OF PATIENTS

The diagnosis of splenic lymphoma with villous lymphocytes (SLVL) depends on more than 30% of circulating lymphocytes having the characteristic morphology of villous lymphocytes (VL) [Bennett et al 1989]. CLL diagnosis, on the other hand, is usually associated with a peripheral blood lymphocyte count of \( >10 \times 10^9/l \). For these reasons the 93 patients were divided into 4 groups to facilitate analysis.

**Group A** - Villous lymphocytes \( >30\% \). PBLC \( >10 \times 10^9/l \)
10 patients (11%)

**Group B** - Villous lymphocytes \( >30\% \). PBLC \( <10 \times 10^9/l \)
20 patients (22%)

**Group C** - Villous lymphocytes 15-29\%. PBLC \( <10 \times 10^9/l \)
12 patients (13%)

**Group D** - Villous lymphocytes \( <15\% \). PBLC variable
51 patients (54%)

CLINICAL FEATURES

Age and sex distribution
These results are shown in table 1. Patients with PBLC \( >10 \times 10^9/l \) and \( >30\% \) villous lymphocytes (ie. group A) were significantly older than patients in the other three groups.
(p < 0.01). (figure 10). Women are probably over-represented in all four groups because women of reproductive age consulted doctors more often than their male counterparts as a result of pregnancy and were therefore more likely to have their splenomegaly detected. Overall 33% of women in the study were pregnant: group A - 0, group B - 7 (39% of women in group), group C - 2 (18%), group D - 14 (27%). Even if these pregnant women are excluded there is still a predominance of women in most groups (table 2).
Figure 10. Age related to PBLC in patients with >30% VL (ie. groups A and B)

VL = villous lymphocytes
PELC = peripheral blood lymphocyte count
Symptoms
The main presenting symptoms of all patients are shown in table 3. Symptoms were related to splenomegaly (29 patients), fever (22), anaemia (19), anorexia or weight loss (12), jaundice (12), general malaise (7) and cough (6). Other symptoms occurring in individual patients included oedema, thirst (secondary to the onset of diabetes mellitus), diarrhoea, epistaxis, and confusion due to hyperviscosity. In 12 patients (13%) splenomegaly was an incidental finding. Most patients had been aware of a left sided abdominal mass for many years preceding diagnosis (53% for 0 - 5 years, 47% for 5 - 20 years) but it had not been troublesome enough to prompt them to seek medical advice.

Signs
Almost all patients (91/93) had splenomegaly (photograph 4) and the vast majority of these also had hepatomegaly. Lymphadenopathy was present in a few cases but was not a prominent feature.
Photograph 4. Massive splenomegaly in a Ghanaian patient
Group A - High VL, high PBLC
The mean spleen size was 20.7cm (range 8-41, SD 9.8) and all but 1 of these patients also had hepatomegaly (3-15cm). Lymph nodes were only significantly enlarged and generalised in 1 patient; 4 others had minimal lymphadenopathy.

Group B - high VL, low PBLC
The mean spleen size in this group was 15.2cm (SD 3.6). All patients had hepatomegaly which ranged from 4 to 12cm and 3 had minimal lymphadenopathy.

Group C - intermediate VL, low PBLC
11/12 patients had splenomegaly (one had previously had splenomegaly which responded to treatment) (mean 14.8cm, SD 4.6), 11/12 had hepatomegaly and none had palpable lymphadenopathy.

Group D - low VL, variable PBLC
In this group 47/48 patients had splenomegaly (mean 15.4 cm, SD 4.9). 1 woman had previously undergone splenectomy for massive splenomegaly. 2 patients did not have hepatomegaly. 11/46 patients had minimal lymphadenopathy. In 2 patients, both with high PBLC, the nodes were significantly enlarged.

Spleens in group A were significantly larger (p <0.05) than spleens in groups B, C and D. There was no relationship between the number of villous lymphocytes and spleen size in the other groups (figure 11).
Figure 11.

Relationship between spleen size and percentage of villous lymphocytes

Spleen size (cm)

Percentage of villous lymphocytes
HAEMATOLOGICAL RESULTS

White cell values

The mean (SD) white blood cell count in groups A-D was 42.7 x 10^9/l (28.4) in group A, 4.8 x 10^9/l (2.4) in group B, 6.1 x 10^9/l (4.4) in group C and 61.9 x 10^9/l (202, range 2-1240) in group D. As most studies of SLVL refer to the peripheral blood lymphocyte count rather than the total white cell count the PBLC will be used throughout this thesis.

The normal upper level for the peripheral blood lymphocyte count (PBLC) in Caucasians is 4.0 x 10^9/l [Wintrobe 1981]. The equivalent level for Ghanaians is slightly lower (3.2 x 10^9/l) and as this value is more appropriate for this study it will be used to delineate the upper limit for these patients. The lower limit for Ghanaians is 0.5 x 10^9/l [Bruce-Tagoe et al 1977]. The PBLC in groups A-D is depicted in figure 12.

Group A - high VL, high PBLC

PBLC in this group varied from 13-75 x 10^9/l (mean 36.4, SD 23.5).

Group B - high VL, low PBLC

The mean PBLC in this group was 2.2 x 10^9/l (SD 1.5). 20% of patients had PBLC >3.2 x 10^9/l but <10 x 10^9/l. 2 patients had PBLC of 0.3 and 0.4, just below the Ghanaian lower limit of 0.5 x 10^9/l.
**Group C - intermediate VL, low PBLC**

The mean PBLC was $2.7 \times 10^9/\text{l}$ (SD 1.5). 33% had PBLC >3.2 $\times 10^9/\text{l}$ the highest value in this group being $5.3 \times 10^9/\text{l}$.

**Group D - low VL, variable PBLC**

PBLC >$10 \times 10^9/\text{l}$ occurred in 14% patients (mean $272.0 \times 10^9/\text{l}$, SD 330.6), 3.3-$10 \times 10^9/\text{l}$ in 22% patients and <3.2 $\times 10^9/\text{l}$ in 55% (mean $2.8 \times 10^9/\text{l}$, SD 2.0). None were below the normal range. In 5 patients the PBLC was not known.

**Haemoglobin**

The haemoglobin results from this study will be compared to those quoted by Fleming [1984] which were derived from a combination of sources and based on work from tropical areas. These normal tropical values are:

- **Men** - 13g/dl
- **Women (non-pregnant)** - 12g/dl
- **Women (pregnant)** - 11g/dl

The haemoglobin values for each group are depicted in figure 13.
Figure 12. Peripheral blood lymphocyte count in groups A-D

PBLC (x10^9/l)

PBLC = peripheral blood lymphocyte count (excluding patient with PBLC >1000)
Haemoglobin values in groups A-D

There is no statistical difference between groups A-D.

Haemoglobin g/dl (mean +/- SEM)
**Group A - high VL, high PBLC**

The mean haemoglobin value in this group was 8.6g/l (SD 2.6). 9/10 patients had values below the normal tropical value.

**Group B - high VL, low PBLC**

The mean haemoglobin value in this group was 6.5g/l (SD 2.6). All patients had values below normal.

**Group C - intermediate VL, low PBLC**

The mean haemoglobin in this group was 7.2 g/l (SD 3.0). All values fell below the appropriate normal tropical level.

**Group D - low VL, variable PBLC**

The mean haemoglobin in this group was 6.7g/l (SD 2.8). 15/16 men, 15/19 non-pregnant women and 11/12 pregnant women had sub-normal levels.

Overall only 7/87 (8%) patients were not anaemic. There was no statistical difference in the haemoglobin level between any of these groups.

**Bone marrow lymphocytosis**

Bone marrow lymphocytosis was defined as lymphocytes comprising over 23.8% of nucleated bone marrow cells [Wintrobe 1981]. 6/6 patients in group A, 8/16 group B, 6/8 group C and 28/46 from group D (including 5/5 with PBLC >10 x 10⁹/l) had lymphocytic infiltration of their bone marrow (figure 14). It
was not possible to differentiate villous from non-villous lymphocytes in these specimens. The degree of bone marrow infiltration was significantly greater in group A than in the other 3 groups (p <0.01 in each case) but there was no difference between groups B, C and D.
Figure 14.

Percentage of bone marrow lymphocytes in groups A-D

BM = bone marrow

A > B, C or D (p < 0.01 in each case)
Haemoglobin electrophoresis

Certain genetic red cell traits, such as haemoglobin AS, afford protection against severe malaria. Such individuals would be expected to have a lower incidence of disorders with malarial aetiology than people with normal haemoglobin genotype. Malaria may play a role in the pathogenesis of HMS and SLVL. In order to test whether haemoglobin AS heterozygotes were underrepresented any of the patient groups, haemoglobin electrophoresis was carried out in 76 controls and 69 patients. An additional 7 patients were screened for the presence of sickle haemoglobin; 6 were negative (1 group B, 1 group C and 4 group D) and 1 positive (group D). The electrophoresis results are depicted in table 4 and photograph 5. There were too few results available for haemoglobin genotype in patients in group A to allow comparison with the geographically-matched control group. Group D was the only group which had a significantly lower number of patients with haemoglobin AS than the controls (p <0.02), 2% compared to 26% in the controls.

The 21 patients who were considered to have HMS on the basis of a good response to treatment had genotype distribution: 17 AA (80%), 1 AS (5%), 2 SS (10%) and 1 thalassaemia intermedia (5%). The expected number of individuals with AS genotype in this HMS group would have been 5 or 6 whereas only 1 out of 22 had sickle cell trait.
would have been 5 or 6 whereas only 1 out of 22 had sickle cell trait.

Photograph 5. Examples of haemoglobin electrophoresis

Haemoglobin samples applied to cellulose acetate membranes and electrophoresed for 50 minutes at 150V
Electron microscopy of red cells

Red cells from 15 unselected patients, 2 from group A, 3 from group B and 10 from group D, were scanned for the presence of surface pocks. None of these patients had more than 2% of cells exhibiting pocks. This is within the normal range and is therefore not indicative of splenic hypofunction.

Photograph 6. Scanning electron microscopy of red cells showing absence of pocks (x6250)
RESPONSE TO TREATMENT

Although the spleen in HMS does respond to anti-malarial therapy, shrinkage is slow. A reduction in splenic size can usually be detected by about 3 months but the maximum response may not be achieved until 12 months after the commencement of treatment (figure 15). On the other hand, spleens which have enlarged as the result of a malignant process may respond initially to treatment but this effect is not sustained or pronounced (figure 16). 35 patients received treatment with anti-malarial drugs for more than 3 months and so their response to treatment could be analysed (table 5). 25 individuals had a good response, 5 had a partial response and 5 had no response. The relationship between the response to treatment with anti-malarial drugs and the number of villous lymphocytes is shown in figure 17.

**Group A - high VL, high PBLC**
No patients in this group showed a good response to treatment. Two were unresponsive and one had a partial response with 50% reduction in splenic size which was not maintained. This patient was observed for 14 months without showing any clinical or haematological deterioration.

**Group B - high VL, low PBLC**
7/10 patients had a good response with reductions in splenic size of at least 40% maintained for the duration of treatment. 1 patient was completely unresponsive and 2 had partial
responses the spleen showing a reductions of 25-50%. Despite continuing therapy the spleen sizes fluctuated between 20 and 50% of their original size without any evidence of intercurrent infection or haemolysis. These patients have been observed for 9 and 22 months without showing any deterioration in their clinical condition despite this relative lack of response.

**Group C - intermediate VL, low PBLC**

All patients in this group who received adequate anti-malarial medication achieved a good response (5/5). There were no partial or non-responders.

**Group D - low VL, low PBLC**

13 patients had a good response, 2 had no response and 2 had a partial response. Of those with partial responses, in one cases the spleen re-enlarged on treatment and in the other the spleen remained static at 30% of its original size despite over 6 months treatment. This may have been due to an unusually slow response or to partial resistance to therapy.
Figure 15

Good response
Female: age 19

Liver/spleen (cm)

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PG = proguanil  Hb = haemoglobin

Figure 16

Partial response - relapse on treatment
Female: age 40

Liver/spleen (cm)

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Hb (g/dl)

<table>
<thead>
<tr>
<th>PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

PG = proguanil  Hb = haemoglobin
Figure 17. Absolute villous lymphocyte count in each treatment group.

Villous lymphocyte count ($\times 10^9/l$)

- Good response
- Partial response
- No response
Although no patient in group A had a good response to treatment whereas every patient in group C who was followed-up had a good response the small numbers in each group meant that a comparison between response and percentage of villous lymphocytes in each group did not reach statistical significance. Even when the numbers of villous lymphocytes in groups with partial and no response were combined and the compared to those in the good response group the difference just failed to reach statistical significance (p = 0.08).

3 patients, all from group D, received less than 3 months therapy and although they cannot be considered to be good responders, even in the short time of follow-up they demonstrated a marked reduction in spleen size of 19-21%.

**CLINICAL COURSE**

The maximum follow-up time for any patient was 5.5 years; 35 patients have been observed for at least 6 months. This long-term data is available on 10 patients with >30% villous lymphocytes, 4 from group A and 6 from group B, who have been observed for 7 months - 5 years. In group A, 2 patients are well at 9 months and 4.5 years and 2 are deteriorating, one with recurrent pyomyositis and another with diarrhoea and weight loss. The 6 patients in group B have been seen for 9 months - 5 years. 2 are well without any problems, 3 have recurrent haemolysis and 1 has frequent chest infections.
Causes of death

10 patients died during the course of the study, 3 from group A, 3 from group B and 4 from group D. Although the exact cause of death could not be ascertained in many cases as the patients died at home, the most common cause of death appeared to be chest or gastrointestinal infection (7 cases) often complicated by haemolysis. 1 patient died from severe bleeding and the cause of death in the remaining 2 patients was unknown. Details of these cases are given below.

Group A - high VL. high PBLC
A 46 year old male died 2 months after presentation from diarrhoea and dehydration. The other two deaths were in women aged 35 and 58. The younger patient died 11 months after presentation having suffered marked weight loss prior to a terminal chest infection. The older woman had been observed for 26 months during which time she proved to be unresponsive to anti-malarial therapy. She died during an episode of acute haemolysis which had probably been precipitated by infection.

Group B - high VL, low PBLC
The 3 deaths in this group occurred in women aged 30, 32 and 59. Two died within 4 days of presentation from acute haemolysis precipitated by infections. The 59 year old women showed no improvement after 9 months therapy with proguanil and died 30 months after presentation. She had become severely
wasted but the actual cause of death was unknown. Her total white blood count remained normal.

*Group C - intermediate VL, low PBLC*

There were no deaths recorded in this group.

*Group D - low VL, variable PBLC*

1 death occurred in a patient with PBLC >10 x10^9/l. This 42 year old female had had a previous splenectomy and presented with symptoms of hyperviscosity due to a white blood count in excess of 1000 x 10^9/l. She had a good response to crude leukaphoresis but died several months after discharge from hospital. The 3 other deaths in this group were due to chest infections, gastrointestinal infections or malaria.

**SEROLOGICAL RESULTS**

*IgM levels*

The mean IgM level in the 19 individuals in the control group was 1.03g/l (+2SD = 2.05). Mean (SD) IgM estimations in the patient groups were; group A 5.8 (5.3) g/l, group B 5.2 (2.0) g/l, group C 4.7 (2.6) g/l and group D 5.4 (3.7) g/l (figure 18). 88% of all patients had IgM levels over 2SD above the local normal mean, 3/4 from group A, 11/12 from B, 3/4 from C and 28/31 from D.
Figure 18.

IgM levels in patients and controls

IgM g/l (mean +/- SEM)

No difference between groups; B and D significantly higher than controls (p < 0.0001)
IgM levels in groups B and D were significantly higher than in the control group (p <0.0001) and although the IgM levels in group A and C were similar to those in B and D the number of patients was small so the results did not reach statistical significance. The inter-group IgM levels were not significantly different from each other.

One of the diagnostic criteria for HMS is an IgM level greater than 2SD above the local normal mean. Although this value was exceeded in 14/16 patients with HMS (as defined by a good response to proguanil) 26 other patients also had IgM values above this 'diagnostic' level - 2 partial responders, 4 non-responders (figure 19) and 20 with inadequate follow-up data to establish a response pattern. In order to test the high IgM criterion for the diagnosis of HMS it would be necessary to statistically compare the IgM levels in HMS and non-HMS patients with the control group. As some of the 20 patients who had no follow-up may have had HMS this group was not used in this analysis. Only those in whom a partial or non-response was fully documented and in whom IgM levels had been measured were analysed (6 patients) and compared to 16 patients with HMS. Both the HMS and the non-HMS patients had IgM levels that differed significantly from the control value (p < 0.0001 and p = 0.012 respectively) but the IgM values in each of the patient groups did not differ from one another.
IgM levels in treatment response groups and controls

Patients with all grades of response (including those with short follow-up periods) have significant higher IgM levels than controls (p < 0.012)
Serum protein electrophoresis was performed on 30 patients from groups A, B and C (photograph 7). All but two showed polyclonal increase in gamma globulin compared to sera from Western Europeans. In addition, abnormal paraprotein bands were seen in 5 samples (17%); 2/6 from group A (33%), 3/17 (18%) from group B and none from group C. The majority of bands occurred in the gamma region with one in the beta region and one having an oligoclonal pattern. Typing by immunofixation was not possible in all cases due to intense background staining produced by the polyclonal gamma globulins. Typed paraproteins were identified as IgM kappa plus IgG kappa in one patient from group A and IgM k in one patient from group B.
Abnormal paraprotein band in lane 2
Monoclonal antibodies to surface determinants CD2, -4, -8 and -19 were applied to 21 cases in which unfixed blood slides were available and 9 normal, geographically-matched controls (photograph 8). In addition, antibodies to CD11c, CD5 and kappa and lambda were used in patients with high levels of villous lymphocytes. The results for the normal controls are shown in table 6 and those for the patients in table 7.

**Group A - high VL, high PBLC**

In 5/5 patients most cells were positive with antibodies against CD19+ (range 79-96%), cells which were positive with anti-CD2 antibody were in a minority (5-15%). The cells positive for CD5 mirrored the percentage of T cells. No cases exhibited positivity with antibodies against CD11c. Immunoglobulin light chain expression was evaluable in 4 patients and restriction demonstrated in all 4 patients: 3 showed lambda restriction and 1 kappa.

**Group B - high VL, low PBLC**

The cells which were positive with anti-CD19 antibody in 6 patients in this group formed 3-46% and cells which marked positive with antibodies to CD2 formed 41-78%. Light chain restriction was evaluated in 3 individuals and found to be negative in all 3 patients.
Group C - intermediate VL, low PBLC

These results are summarised in table 7. None of 4 patients tested had light chain restriction.

Group D - low VL, variable PBLC

In 5/6 patients from this group the majority of lymphocytes were T cells (ie. CD2+). The majority of cells in one patient who had a high PBLC were B cells (CD19+) with T cells (CD2+) forming only 18% of lymphocytes. The B cells in this case were negative for antibodies to both CD5 and CD11c. Light chain restriction was not evaluable in any of these patients.

Statistical analysis (t tests) were performed comparing each group with the controls. Only group A showed a significant difference in the distribution of surface markers having higher levels of CD19 and lower levels of CD2, -4 and -8 than controls and groups B, C and D. It has been reported that HMS is associated with a raised CD4:CD8 ratio due to a reduction in cells carrying the CD8 marker rather than an increase in cells reacting with anti-CD4 antibodies. A comparison of surface marker distribution was therefore made between HMS patients (ie. those with a good response to anti-malarial therapy) and the control group. There was no difference between the HMS patients and controls for surface marker distribution. Although the CD4:CD8 ratio in HMS was 2.76 and in controls was 1.8 this difference did not reach statistical significance.
Photograph 8. Lymphocytes positive with antibody to CD19 determinant
DNA for studies of immunoglobulin gene rearrangements was available in 28 patients, 8 of whom had abnormal bands after autoradiography of Southern blots (photograph 9).

**Group A - high VL, high PBLC**
All patients tested (5/5) had JH rearrangements and 0/3 had BCL-1 rearrangements.

**Group B - high VL, low PBLC**
1/8 individuals in this group had a JH rearrangement. This was a 42 year old woman with a normal white blood count. She was not followed-up so no firm diagnosis was made and there were no data available concerning her lymphocyte phenotype.

**Groups C and D - intermediate/low VL, low/variable PBLC**
2/2 patients from group C and 10/12 from group D had no rearrangements. J rearrangements were found in a 62 year old man with no firm diagnosis and a PBLC of $104 \times 10^9/l$ and a 40 year old woman with a normal PBLC and a slight response to proguanil.
Photograph 9. Southern blots showing clonal immunoglobulin gene rearrangements in 8 patients

10μg of Hind III digested DNA were probed for J^H. Clonally rearranged bands are shown in 5 patients from group A (lanes 1-5), 1 from group B (lane 6) and 2 from group D (lanes 7 and 8). Lane 9 represents the normal pattern seen in a polyclonal lymphocyte population.
Table 1. Age and sex distribution in groups A-D

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Males</th>
<th>Females</th>
<th>Mean age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>52.9 (35-66)</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>2</td>
<td>18</td>
<td>32.9 (15-75)</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>1</td>
<td>11</td>
<td>35.6 (18-65)</td>
</tr>
<tr>
<td>D</td>
<td>53</td>
<td>19</td>
<td>34</td>
<td>35.4 (15-75)</td>
</tr>
</tbody>
</table>

Group A older than groups B, C or D (p < 0.01)

Table 2. Sex distribution in groups A-D (excluding pregnant women)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>Males</th>
<th>Females</th>
<th>M:F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>0.42</td>
</tr>
<tr>
<td>B</td>
<td>13</td>
<td>2</td>
<td>11</td>
<td>0.18</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>1</td>
<td>9</td>
<td>0.11</td>
</tr>
<tr>
<td>D</td>
<td>37</td>
<td>20</td>
<td>17</td>
<td>1.12</td>
</tr>
</tbody>
</table>
Table 3. Presenting symptoms

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenic discomfort</td>
<td>7</td>
<td>6</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Anorexia</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Anaemia</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Jaundice</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Cough</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Malaise</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

(Some patients complained of more than one symptom)

Table 4. Results of haemoglobin electrophoresis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(76)</td>
<td>(6)</td>
<td>(16)</td>
<td>(10)</td>
<td>(44)</td>
</tr>
<tr>
<td>AA</td>
<td>65%</td>
<td>100%</td>
<td>81%</td>
<td>67%</td>
<td>84%</td>
</tr>
<tr>
<td>AS</td>
<td>26%</td>
<td>0%</td>
<td>19%</td>
<td>22%</td>
<td>2%</td>
</tr>
<tr>
<td>AC</td>
<td>6%</td>
<td>0%</td>
<td>0%</td>
<td>11%</td>
<td>2%</td>
</tr>
<tr>
<td>Others (SS/SC/CC/thal)</td>
<td>3%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>12%</td>
</tr>
</tbody>
</table>

Genotype AA higher than expected in groups A plus B (p < 0.05)
Table 5. Treatment responses in groups A-D

<table>
<thead>
<tr>
<th>Response</th>
<th>Group A (3)</th>
<th>Group B (10)</th>
<th>Group C (5)</th>
<th>Group D (17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good</td>
<td>(25) 0%</td>
<td>70%</td>
<td>100%</td>
<td>76%</td>
</tr>
<tr>
<td>Partial</td>
<td>(5) 33%</td>
<td>20%</td>
<td>0%</td>
<td>12%</td>
</tr>
<tr>
<td>None</td>
<td>(5) 67%</td>
<td>10%</td>
<td>10%</td>
<td>12%</td>
</tr>
</tbody>
</table>

Table 6. Surface marker studies in 9 Ghanaian controls

<table>
<thead>
<tr>
<th></th>
<th>CD2</th>
<th>CD4</th>
<th>CD8</th>
<th>CD19</th>
<th>CD4:CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>48</td>
<td>28</td>
<td>10</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>38</td>
<td>15</td>
<td>8</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>54</td>
<td>58</td>
<td>44</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>50</td>
<td>28</td>
<td>62</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>40</td>
<td>18</td>
<td>16</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>38</td>
<td>27</td>
<td>30</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>28</td>
<td>23</td>
<td>7</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>52</td>
<td>16</td>
<td>25</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>38</td>
<td>30</td>
<td>24</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>69.1</td>
<td>42.9</td>
<td>27.0</td>
<td>25.1</td>
<td>1.8</td>
</tr>
<tr>
<td>SD</td>
<td>(6.5)</td>
<td>(8.6)</td>
<td>(12.9)</td>
<td>(18.3)</td>
<td>(0.75)</td>
</tr>
</tbody>
</table>
Table 7. Surface marker studies in patients
(mean (SD), range)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>CD2</th>
<th>CD4</th>
<th>CD8</th>
<th>CD19</th>
<th>CD5</th>
<th>CD11c</th>
<th>CD4:8</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>9.6 (4.6)</td>
<td>4.0 (2.5)</td>
<td>4.6 (5.6)</td>
<td>86.0 (6.8)</td>
<td>10.4 (4.2)</td>
<td>6.2 (4.8)</td>
<td>0.9 (0.7)</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>60.8 (15.4)</td>
<td>35.2 (9.0)</td>
<td>20.8 (11.0)</td>
<td>15.3 (15.6)</td>
<td>53.6 (5.2)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>63.3 (10.4)</td>
<td>45.3 (21.4)</td>
<td>16.3 (8.4)</td>
<td>6.0 (5.1)</td>
<td>58.8 (27.3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>66.2 (5.8)</td>
<td>41.0 (17.2)</td>
<td>27.6 (15.6)</td>
<td>10.6 (5.2)</td>
<td>56.8 (29.0)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Pattern of surface markers significantly different from controls in group A (p <0.001 for CD2, -4, -8 and -19). Groups B, C and D similar to controls.

ND = not done

Table 8. Immunoglobulin gene rearrangements

<table>
<thead>
<tr>
<th>Group</th>
<th>Total</th>
<th>J rearranged</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>13</td>
<td>2</td>
</tr>
</tbody>
</table>
Ten Ghanaian patients have been described in this study who could clearly be diagnosed as splenic lymphoma with villous lymphocytes (SLVL). They had splenomegaly, lymphocytosis and typical circulating lymphocytes with polar villi. The monoclonal nature of SLVL was confirmed by a combination of factors such as serum paraproteins, restricted immunoglobulin light chain expression and clonal rearrangements of the immunoglobulin genes. A further twenty patients partially fulfilled current criteria for SLVL by having splenomegaly and at least 30% villous lymphocytes in their peripheral blood. However, monoclonality could not be demonstrated in the majority of these individuals and they did not have a marked lymphocytosis. These 30 patients, who comprise groups A and B, were selected from a group of 93 patients with splenomegaly due to various causes, predominantly HMS or African 'CLL'.

African CLL - a misnomer

The existence of SLVL in tropical West Africa may partly explain why 'CLL' in Africa has been reported to be associated with splenomegaly and not lymphadenopathy and to affect two distinct groups, middle-aged women and elderly men. In the light of the findings of this study it now seems possible that some of the younger patients actually had other forms of B
cell lymphoma, such as SLVL. The frequency of classical CLL with lymphadenopathy, hypogammaglobulinaemia and CD5+ lymphocytes in West Africa is not known. The realisation that SLVL in Ghana may be mistaken for CLL has important management implications. Normal first-line treatment for CLL includes chlorambucil although this may not be readily available in tropical countries. Chlorambucil is not appropriate treatment for SLVL which instead, responds well to splenectomy. Thus in the case of these two disorders it is essential to make the correct diagnosis.

**Differentiation of SLVL from CLL in this study**

This study shows that although diagnoses of SLVL and CLL in Ghana are both made in the presence of splenomegaly they can be distinguished by differences in lymphocyte morphology. Typical villous lymphocytes are present in Ghanaian patients with SLVL but not with CLL. SLVL is shown to predominantly affect women over 40 years (table 1) and has similar symptomatology to CLL (table 3), patients either complaining of generalised symptoms or splenic discomfort. Anaemia is common in both SLVL and CLL and the white count usually exceeds $10 \times 10^9/\text{l}$. However, the peripheral blood lymphocyte count tends to be lower in these Ghanaian patients with SLVL than in CLL.

I have shown SLVL to be associated with IgM levels 4-5 times
greater than the local, normal mean. Almost every patient tested had a polyclonal increase in gamma globulins and, superimposed on this, about 20% of cases had a paraprotein band in their serum. Although there are no large studies of immunoglobulin levels in African CLL, raised gammaglobulins are not a common feature of lymphoproliferative neoplasms. CLL in particular, is usually associated with immune suppression rather than with increased gamma globulins. Monoclonal IgM bands have been reported in 5% of B-CLL and 10% of lymphocytic lymphomas in North America [Alexanian 1975]. This frequency of raised IgM is one hundred times higher than in normal populations and other cancers.

The majority of lymphocytes in cases of CLL in Europe and North America are positive for antibodies for CD5. There is a paucity of data on the lymphocyte phenotype in African CLL and information from Caucasian CLL cases cannot necessarily be extrapolated to CLL in tropical Africa. In view of the different demographical and clinical features of CLL in these two geographical areas it is possible that they are in fact different entities. I have demonstrated by surface marker studies that SLVL in Ghana is a B-cell disorder negative for antibodies to the CD5 determinant. In addition the study identified in group D | a case of B-lymphoproliferative disorder associated with splenomegaly and no lymphadenopathy. Villous lymphocytes were not a prominent feature and the lymphocytes were negative for antibodies to the CD5 determinant making CLL an unlikely diagnosis.
In Essien's study of CLL in Nigeria [1976], 63% of patients with CLL died within 3 months of presentation and 90% had died by 3 years despite the administration of "appropriate chemotherapy". 15% died from infection and 3.3% had lymphoblastic transformation. 2 patients who had white counts $<50 \times 10^9/l$ were considered to be "in remission" 5 and 7 years after initial treatment with chlorambucil. I also found the course of SLVL to be variable with 6 patients out of 30 with over 30% villous lymphocytes dying during the period of the study, usually from infections.

**Differentiation of SLVL from HMS in this study**

As with CLL, this study shows that both SLVL and HMS affect women more than men but SLVL tends to occur in a slightly older age group (table 1). Clinically, HMS and SLVL proved difficult to distinguish; SLVL patients seem to have a greater tendency to develop moderate lymphadenopathy and, on average, have larger spleens than patients with lower numbers of villous lymphocytes (figure 11). These variations are not helpful in the diagnosis of individual patients. Both SLVL and HMS present with non-specific symptoms and hepatosplenomegaly (table 3). Jaundice due to acute haemolysis appears to be more common among HMS patients, particularly those who are pregnant [Hamilton 1966], than in SLVL patients.

Haemoglobin levels are shown to be similarly reduced in both
HMS and SLVL and I have demonstrated that the total white blood count is usually raised in SLVL and associated with a significantly greater degree of bone marrow involvement than HMS. Nevertheless, 8-70% of African HMS patients may show a blood and bone marrow lymphocytosis [Lowenthal et al 1980, Bryceson et al 1976]. This phenomenon of lymphocytosis in HMS has not described from other parts of the world. IgM levels in HMS are stated to be at least 2 SD above the local normal mean in the diagnostic criteria published in 1981 [Fakunle]. My findings generally confirmed this but it was also noted that such elevations of IgM were not confined to HMS; markedly raised IgM levels were also found in patients with SLVL and splenomegaly due to other causes. These findings support those of DeCock et al [1986] who looked specifically at the IgM criterion by comparing two groups of Kenyan patients with HMS. They found no clinical difference between those with IgM concentrations 2SD above the local normal mean and the rest and concluded that it was "unreasonable" to consider this degree of IgM increase as essential for the diagnosis. Studies from Malawi [Molyneaux et al 1979] and Uganda [Ziegler et al 1973] have also found that HMS is underdiagnosed if the 2SD IgM criterion is a prerequisite. Raised IgM levels are therefore not specific for HMS and so the usefulness of this criterion in the diagnosis of HMS needs to be re-evaluated. An update of the criteria for HMS diagnosis is probably indicated at this time, at least for scientific purposes, to include a method of excluding monoclonal disease.
Until this study was carried out there was very little data published on the lymphocyte phenotype in HMS. I have found that the lymphocyte surface antigen determinants in HMS and SLVL patients with normal lymphocyte counts are no different from those in the normal population. One of the major distinguishing features between SLVL and HMS proved to be that SLVL shows evidence of monoclonality, such as immunoglobulin light chain restriction and immunoglobulin gene rearrangements, whereas in HMS the lymphocytes are polyclonal.

**Susceptibility to infections**

Although some patients in this study have only been followed for a few years it is clear that the course of SLVL is variable, some patients succumbing to infections shortly after presentation and others remaining asymptomatic for long periods. Infections, which were the terminal event in most of the patients who died during this study, are the most common cause of death generally in developing countries. There is no evidence from my work that patients with SLVL and other causes of splenomegaly are particularly prone to infections either as a result of splenic hypofunction or hypogammaglobulinaemia.

The natural history of HMS in Papua New Guinean patients has been described by Crane [1981]. The patients progressed from being minimally disabled to being completely incapacitated as the spleen enlarged. A suprisingly high mortality rate of 63% in 18 years was found [Crane 1986a] amongst a group of 148
Watuts with untreated HMS from Papua New Guinea; most of these deaths were due to infections. Not surprisingly, reports in the literature about the infection risks in HMS are contradictory because infections are such a common cause of death even amongst previously healthy individuals in developing countries. An impaired neutrophil response to pyogenic infection has been observed in HMS patients [Hewlett and Pitchnmoni 1987] manifest by an abnormally slow reduction in temperature after treatment for pneumonia despite normal resolution of radiological abnormalities [Awunor-Renner 1979].
COMPARISON OF SLVL IN GHANA AND ELSEWHERE

Similarities

A summary of these comparisons is given in table 9.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Ghana</th>
<th>Elsewhere</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex ratio (M:F)</td>
<td>1:5</td>
<td>2:1</td>
</tr>
<tr>
<td>Peak incidence (years)</td>
<td>35-45</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>100%</td>
<td>80-100%</td>
</tr>
<tr>
<td>Other clinical features</td>
<td>Similar</td>
<td>Similar</td>
</tr>
<tr>
<td>Anaemia</td>
<td>Common</td>
<td>Common</td>
</tr>
<tr>
<td>Serum paraproteins</td>
<td>20%</td>
<td>25-50%</td>
</tr>
</tbody>
</table>

Clinical features

This study shows that the same symptoms were experienced by SLVL patients in Ghana as in more developed countries; discomfort related to splenic growth or general feelings of malaise were the most common presentations. Many patients were asymptomatic and had splenomegaly detected incidentally. Splenomegaly of at least 8cm was found in every SLVL patient in this study; as in temperate regions, lymphadenopathy was
not a prominent feature in Ghana whereas hepatomegaly was almost universal (table 3).

Haematology

Anaemia was found to be a common feature of SLVL in Ghana (figure 13). Erythrocyte and bone marrow appearances of these and other patients from West Africa [Fakunle 1981, Watson-Williams and Allan 1968] indicated that this was due to a combination of iron and folate deficiency, lack of vitamin B12 being unusual in Africans. The anaemia may have been caused by a combination of poor nutrition, hookworm and malaria infections exacerbated by premature destruction of red cells in the enlarged spleens.

The typical appearance of the villous lymphocytes in Ghana was the same as that described in western countries. The morphological appearance of cells from several of these Ghanaian patients was confirmed as being compatible with SLVL by Prof D Catovsky of The Royal Marsden Hospital, London.

Serology

I identified serum paraproteins in 20% of Ghanaian patients with >30% villous lymphocytes. This is similar to the incidence of paraproteins in the serum of Caucasians with SLVL although if urine is also examined up to 60% of individuals can be shown to have monoclonal bands. The incidence may
therefore have been higher in Ghana if urine had been also
been examined. Paraproteins were more difficult to detect in
these tropical patients than in those from temperate areas
because the high levels of immunoglobulins produced a densely
staining background smear in which a faint paraprotein band
was difficult to detect. It may therefore be helpful to use a
highly sensitive technique such as immunofixation to detect
small levels of paraprotein even in the face of a normal
electrophoretic pattern.

Differences

Prevalence

The fact that I identified 10 patients with SLVL single-handed
during a 4 year period in Ghana implies that the disorder must
be fairly common. Although there are no figures available for
the prevalence of SLVL in temperate regions, it appears to be
an uncommon disorder among Caucasians. Mulligan and co-workers
[1991] describe 50 patients that they saw over 15 years.
Genetic factors may be responsible for this geographical
difference in prevalence but there are no reports of SLVL
occurring more frequently in US Blacks than Whites. It is
therefore more likely that environmental influences such as
infections, especially malaria, and poor sanitation play a
part in the aetiology of SLVL in Ghana.
Sex and age distribution

This study demonstrated that these parameters differed markedly from the distribution that occurs in developed countries (table 1). In Ghana there was a predominance of women, the male:female ratio in these 30 patients being 1:5, whereas in Caucasians men are affected twice as often as women. As discussed earlier, women of reproductive age may be over-represented in this study but even in patients beyond reproductive age with PBLC over $10 \times 10^9/\ell$ the ratio is 1 male:2 females. Women do not seem to be especially at risk of developing other lymphoproliferative diseases in the tropics: for example, the male:female ratio in Burkitt's lymphoma is 2:1 [Fleming 1985]. It has been suggested that the reason CLL affects younger women in Africa is related to the transmission of an oncogenic agent to which women of reproductive age are more susceptible because of the physiological immune depression which occurs during normal pregnancy [Fleming 1988].

I have shown that the peak incidence of SLVL in Ghana appears to be 2 decades younger than in Caucasians. This may reflect the younger age structure of the Ghanaian population and the presence of different aetiological factors, possibly environmental or genetic, which induce the development of lymphoma earlier in Ghana.
White cell counts

The total white blood count and PBLC in SLVL in Ghana are lower than elsewhere (figures 9, 10 and 12) with a wider range (0.5-75.0 in Ghana compared to 2.0-40.0 x 10⁹/l elsewhere) [Melo et al 1987, Spriano et al 1986]. Only 15% of Caucasians have white cell counts less than 10⁹/l whereas in this study 61% had total white blood counts of 1.3-7.5 x 10⁹/l. This is probably a function of the mode of selection of these tropical patients. By concentrating on patients with HMS, I have identified a group of individuals who are targets for the development of SLVL.

These Ghanaian patients also had a bone marrow lymphocytosis, the degree of marrow involvement being independent of their peripheral lymphocyte count. As trephine biopsies were not performed it was not possible to determine the pattern of lymphocytic infiltration although previous reports from Europe suggest that it in SLVL it may be nodular or diffuse.

THE DIFFERENTIAL DIAGNOSIS OF SLVL

There are several examples in the literature of the diagnostic confusion posed by African cases of splenomegaly with lymphocytosis but without the immuneparesis usually associated with CLL. To date the vast majority of reports of CLL from Africa stress that it most commonly presents with splenomegaly and often affects younger women. HMS also occurs in this age
group and may be associated with a lymphocytosis.

I also experienced difficulty in categorising disorders characterised by splenomegaly with or without a raised lymphocyte count in Ghana. Without recourse to sophisticated technology it is often not possible to conclusively diagnose HMS or CLL and related disorders in tropical hospitals. These diagnostic difficulties are illustrated by two reports from opposite sides of the continent. Bagshawe [1970] suggested that in Kenya "the variable response to proguanil may indicate that more than one disease process has been included within the diagnosis of tropical splenomegaly". In the same year Sagoe in Nigeria reported that "cases of malignant lymphoma presenting with gross splenomegaly .... have been misclassified as tropical splenomegaly syndrome. Cases of tropical splenomegaly syndrome with a lymphocytic leukaemoid reaction .... have been misclassified as CLL".

On review of the literature it is now clear that many of these "misclassified" cases could in fact have been SLVL although not enough detail was given about lymphocyte morphology to confirm this. Examples of such instances and their location are given below.
Nigeria - Allan and Watson-Williams [1963]
In a study of CLL from Nigeria it was noted in 9 patients out of 22 that chlorambucil produced a reduction in the white cell count but had no effect on the spleen size, haemoglobin or platelet count until combined with proguanil. Proguanil alone produced significant improvement in these parameters in a further 3 patients with splenomegaly and a high white cell count.

Nigeria - Bryceson [1976]
During a survey of splenomegaly in Northern Nigeria, Bryceson described a non-neoplastic syndrome he called gross lymphoid hyperplasia. This occurred in multiparous Hausa women and was generally characterised by a lymphocyte count >30 x 10^9/l and normal immunoglobulin levels. The disease responded to proguanil and was therefore thought by later researchers to represent a variant of HMS [Hewlett and Pitchnmoni 1987]. Despite the use of strict criteria for the diagnosis of HMS, CLL and gross lymphoid hyperplasia, Bryceson was not able to make a definitive diagnosis in 13/75 patients with splenomegaly in northern Nigeria. They were thought not to have HMS because they either failed to respond to proguanil, had evidence of immune-suppression (low PHA-stimulated lymphocyte transformation) or did not have hepatic sinusoidal lymphocytosis.

Nigeria - Fakunle [1979]
Fakunle reported 4 patients with massive splenomegaly, hepatic
sinusoidal lymphocytosis and absolute lymphocyte counts in excess of $34 \times 10^9/l$. They also had IgM levels over 19.6g/l (local normal mean 2.95g/l). The illness was thought to be HMS with a leukaemoid reaction because the immunoglobulin was polyclonal with both kappa and lambda light chains and only 5% of CLL cases have high IgM levels [Preud'homme 1972]. However, all these features, including the raised gammaglobulin are compatible with a diagnosis of SLVL.

**Nigeria - Williams [1983]**

In a study of the incidence of lymphoma sub-types in urban West Africans, CLL was diagnosed by a white blood count over $10 \times 10^9/l$ and tissue invasion. A bimodal age incidence was noted, and in those patients aged less than 50 years the sex ratio was 1 male:6 females. The author suggested that in Nigeria there were two forms of CLL. The younger patients were of low socioeconomic status and had splenomegaly. This female excess has only been noted in tropical Africa suggesting that environmental factors might be partly responsible. However, the disease also occurs in young US Blacks but not in Caucasians implying there may be a genetic influence in these younger patients (figure 1).

**Nigeria - Ukaejiofo [1987]**

Ukaejiofo placed less reliance than Fakunle [1979] on raised immunoglobulins and diagnosed CLL in 8 patients with similar clinical presentations to those described by Fakunle, on the basis of absolute lymphocyte counts of at least $31.3 \times 10^9/l$
and polyclonal IgM levels of 0.72-8.6g/l (local normal range 0.5-4.0g/l).

Congo - Sonnet [1967]
A distinction has been made between two different groups of Congolese Bantus with CLL. One group had classical CLL with lymphadenopathy and white blood counts of 53-560 x 10^9/l. The other had splenomegaly rather than adenopathy, a lower white blood count (4-50 x 10^9/l) and hypergammaglobulinaemia with monoclonal bands. All patients were aged 45-65 years with M:F ratio of 2:1. In retrospect the second group may have been SLVL but insufficient information was provided concerning the lymphocyte morphology to indicate this diagnosis.

Gabon - Perrett [1991]
Primary splenic lymphoma is uncommon and has been defined by DasGupta [1965] as disease confined to the spleen or hilar nodes with no recurrence for at least 6 months after splenectomy. Two cases have been reported from Gabon; both had diffuse, large-cell histology. The incidence of primary splenic lymphoma was thought to be underestimated in Africa because it may have been mistaken for HMS. The lack of peripheral blood involvement in these cases does not exclude a diagnosis of SLVL as in some cases the peripheral blood lymphocyte count is normal.
EVIDENCE FOR MONOCLONALITY OF B-LYMPHOCYTES IN SLVL

I could only confirm the monoclonal nature of the lymphocytes in SLVL in patients with raised lymphocyte counts. Both light chain restriction and immunoglobulin gene rearrangements were found in all Ghanaian patients with high PBLCs that were tested. Interestingly, monoclonality was only found in 1/8 patients with >30% villous lymphocytes and a PBLC <10 x 10^9/l. The other 7 patients in whom studies of light chain restriction (3) or immunoglobulin gene rearrangements (7) were performed did not have a detectable population of monoclonal cells. Neither of these techniques is very sensitive; light chain restriction interpretation proved to be difficult when unwashed cells were used and Southern blotting cannot detect less than 1 in 10^2 clonal cells. In the absence of demonstrable clonal cells the presence of large numbers of villous lymphocytes is not sufficient on its own to diagnose SLVL in tropical patients. These cells probably represent expansion of a morphological sub-type which is polyclonal as Southern blotting would be expected to detect the presence of a clonal population which comprised a third of the circulating lymphocytes.

The patients in group A with high PBLC (figure 9) clearly have a monoclonal lymphoproliferative disorder (SLVL) which is resistant to treatment with anti-malarial therapy. The patients in group B, all of whom have PBLC <10 x 10^9/l (figure 9), have morphological features of SLVL, usually without
detectable monoclonality, and have responses to proguanil which vary from complete sensitivity to unresponsiveness. Although females predominate in both groups the patients in group B are younger, have less lymphadenopathy and undetectable levels of monoclonal lymphocytes compared to those in group A (tables 1 and 3). These observations suggest that this group of patients with high numbers of villous lymphocytes demonstrates the early and possibly, pre-malignant, stage of SLVL. A careful follow-up of the molecular characteristics of the lymphocytes in this group of patients may clarify the role of HMS in the aetiology of SLVL. If HMS is involved in the pathogenesis of SLVL the first appearance of characteristic villous lymphocytes in the peripheral blood may be an important marker for the development of a lymphoma.

Individual patients with >30% villous lymphocytes in whom serological, phenotypic and molecular data is available

A complete set of data is available for 6 patients from groups A and B four of whom have some evidence (either paraprotein or clonal immunoglobulin gene rearrangement) for a monoclonal process (table 10).
Table 10. Patients with >30% villous lymphocytes in whom a full set of serological, phenotypic and molecular data is available.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sex</th>
<th>PBLC (x10⁹/l)</th>
<th>Paraprotein</th>
<th>CD19+</th>
<th>J⁺RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>F</td>
<td>19.0</td>
<td>YES</td>
<td>96</td>
<td>YES</td>
</tr>
<tr>
<td>51</td>
<td>F</td>
<td>72.0</td>
<td>NO</td>
<td>92</td>
<td>YES</td>
</tr>
<tr>
<td>75</td>
<td>M</td>
<td>0.7</td>
<td>YES</td>
<td>13</td>
<td>NO</td>
</tr>
<tr>
<td>35</td>
<td>F</td>
<td>2.3</td>
<td>YES</td>
<td>10</td>
<td>NO</td>
</tr>
<tr>
<td>35</td>
<td>F</td>
<td>2.5</td>
<td>NO</td>
<td>46</td>
<td>NO</td>
</tr>
<tr>
<td>42</td>
<td>F</td>
<td>1.3</td>
<td>NO</td>
<td>6</td>
<td>NO</td>
</tr>
</tbody>
</table>

PBLC = peripheral blood lymphocyte count
J⁺RA = clonal immunoglobulin gene rearrangement

One patient, who had a PBLC of 19 x 10⁹/l and B-cells forming over 90% of lymphocytes, had evidence of monoclonality with both a serum paraprotein and a clonal immunoglobulin gene rearrangement. Two patients had no demonstrable monoclonal cells by either criterion, two had only a monoclonal protein and one had only a clonal immunoglobulin gene rearrangement. The absence of detectable clonal immunoglobulin gene rearrangements in circulating cells does not necessarily imply that the underlying disorder is not clonal. The malignant cells could be located outside the bloodstream, for example in the spleen, and remain undetected by the methods used in this study to screen the peripheral blood. However, malignant cells which are contained within an organ may still secrete monoclonal proteins into the...
bloodstream so the detection of a serum paraprotein scans for a malignant clone of lymphocytes anywhere in the body. 1 1

Patients with villous lymphocytes between 15 and 29%

12 individuals in this study had 15-29% circulating villous lymphocytes (group C). Again women were preferentially affected and the mean age was similar to that of group A (table 1). Apart from the lack of lymphadenopathy, symptoms (table 3) and signs were also similar and no individual had PBLC over 10 x 10⁹/l. All cases adequately treated with antimalarials showed a good response and monoclonality could not be demonstrated either by light chain restriction or immunoglobulin gene rearrangements.

Patients in this group were initially thought to have HMS and this was confirmed by a good response to treatment in 5/5 and high IgM levels a further 1 patient. It has been hypothesised that HMS may be a pre-malignant disorder; the most likely candidate for a disorder into which HMS might evolve was thought to be African CLL [Fakunle 1981]. Until now, there has been no evidence to support such a theory of lymphomagenesis. Although the normal counterpart of the villous lymphocyte has not been identified it is presumably an activated stage of B-cell lymphopoesis. As I have identified patients with large
numbers of circulating villous lymphocytes but no evidence of monoclonality it is possible that polyclonal expansion of villous lymphocytes may be a necessary early step in the process of lymphomagenesis.

**Problems of diagnosis and management of SLVL in the tropics**

It is very important to be able to differentiate between HMS and SLVL in Africa because the treatment for each condition is different. HMS responds well to anti-malarial prophylaxis and SLVL to splenectomy which may be curative in early disease. In Ghana, patients with at least 30% villous lymphocytes, a high PBLC and a poor response to proguanil can clearly be diagnosed as SLVL even under field conditions. Problems arise when trying to categorise patients with lower numbers of villous lymphocytes or white blood cells and undocumented or equivocal responses to treatment. Where facilities for assessing cellular clonality are available a distinction between SLVL and HMS may still be made but in a tropical setting further evaluation of these conditions will not usually be possible. In the past, phytohaemagglutination-induced blastic transformation of lymphocytes has been used to distinguish between HMS and CLL [Sagoe 1970] but this method is not specific and is not widely available in Africa. This study has shown that some patients have features of both HMS and SLVL having a proguanil sensitive disorder and yet with circulating >30% villous lymphocytes. In practice, differentiation between HMS and SLVL depends on documenting a
good or poor response to anti-malarial therapy which in turn requires adequate long-term follow-up. At least 6 months treatment is needed before a 'non-responder' can be identified and a diagnosis of splenic lymphoma made. During this time the malignant disease may have progressed so that splenectomy may no longer be helpful. An alternative approach might be to recommend splenectomy initially for all HMS patients with high numbers of villous lymphocytes but splenectomy in the tropics carries many risks especially as the vast majority of these spleens measure over 10cm [Bates 1992]. Even after removal of the spleen in HMS the IgM levels continue to rise implying that the underlying pathological process is unaltered. Although this study suggests that HMS may be a pre-malignant disorder it does not provide the direct proof that would be needed to justify splenectomy in all these cases. The benefits and risks of drug treatment and splenectomy are discussed below.

**Anti-malarial prophylaxis**

Anti-malarial therapy for HMS was first tried in Nigeria in 1968 [Watson-Williams and Allan]. It results in a very significant reduction in splenic size and IgM level, an increase in haemoglobin and an improvement in symptoms [Stuiver 1974] all of which deteriorate if therapy is withdrawn. Since no present day anti-malarial agent acts against mosquito-inoculated sporozoites, malaria chemoprophylaxis prevents the development of disease rather
than malaria itself.

There is very little published data concerning long-term usage of anti-malarial drugs and almost none concerning their use in semi-immune populations as in HMS.

**Chloroquine**

Resistance to chloroquine has been reported from almost all parts of the world (except Central America, Haiti, North Africa and parts of the middle East) including Ghana. Nevertheless it continues to be the mainstay of short-term prophylaxis; it is only administered weekly and, despite resistance, it still reduces the severity of infection even though complete suppression may not occur [Weststeyn and DeGeus 1985]. It has a high affinity for melanin-containing tissues of the skin and eye and may cause pruritus in 50-60% of Africans. Its most serious adverse effect is irreversible retinopathy the risk of which increases as the total lifetime dose exceeds 100g of base [Keystone 1990]. This is estimated to be equivalent to about 5 years of prophylaxis at normal doses (300mg base weekly). For both these reasons chloroquine is not suitable for long-term management of HMS. It is safe in pregnancy.

**Proguanil**

This is a biguanide which acts as a dihydrofolate reductase inhibitor. It is a prodrug which acts through its primary
metabolites cycloguanil and chlorcycloguanil. It is one of the best tolerated anti-malarial drugs and is safe in pregnancy at 100mg/day (although folate supplements are needed). It is devoid of serious side effects and cheap and is therefore the most appropriate drug for lifelong use in HMS. The majority of studies of proguanil use have been conducted in non-immune populations visiting malarious areas for less than 5 years. There are very few reports of its long-term use in semi-immune individuals and in the management of HMS. It was given to HMS patients for 1-26 months in Kenya [Bagshawe 1970] and 14-80 months in Nigeria [Watson-Williams and Allan 1968] without any serious adverse effects being reported.

Other prophylactic anti-malarial drugs

Many of these drugs are associated with side effects that preclude their use for long periods. Pyrimethamine/sulphadoxine can produce severe cutaneous reactions and pyrimethamine/dapsone causes a reduction in all blood components, rarely progressing to agranulocytosis. Mefloquine can cause neuropsychiatric complications and its safety in pregnancy has not been established. Side effects of doxycycline include gastrointestinal disturbances, phototoxicity and vaginal candidiasis and it is contraindicated in pregnancy because of tooth discoloration and dysplasia and inhibition of bone growth in the fetus.
Splenectomy in the tropics

In the tropics, the lack of follow-up data precludes an accurate assessment of all the risks of splenectomy. In Uganda, elective splenectomy for patients with hypersplenism and spleens weighing 1.5-4.5kg had an early post-operative mortality of 4.8% [Paliwoda and Hutt 1967]. The danger of splenectomy in the tropics is twofold. Firstly, splenomegaly results in thrombocytopenia, while the size of the spleen may make the procedure technically awkward; platelet and blood transfusions may be required peri-operatively. The high prevalence of HIV and hepatitis B infections has rendered blood transfusion hazardous in tropical areas. Secondly, post-splenectomy patients are particularly susceptible to bacterial infections, especially with encapsulated organisms. This produces the classical syndrome of pneumococcaemia, disseminated intravascular coagulation and shock and has a mortality of 50-80% in established cases in the tropics [Ihkewaba 1988]. In malarious areas, there is the additional potential hazard of overwhelming malaria infection. Vaccination against H influenzae type b, N meningitides and S pneumoniae is indicated prior to elective splenectomy and for those patients who have already undergone splenectomy. Post-operatively, patients require prolonged anti-malarial prophylaxis and urgent empirical therapy for any febrile illness. Research with animal models suggests that partial preservation of the spleen, with arterial structures and splenic architecture intact may be preferable to total
splenectomy [Bowdler 1990]. In tropical Africa the risks of splenectomy usually outweigh any benefits so the procedure should only be undertaken as a last resort.

**HMS AS A TARGET FOR SLVL**

The findings of this study suggest that HMS patients may form a target group which favours the development of this particular type of splenic lymphoma. In order to suggest a plausible sequence of events for this process it is first necessary to review what is known about the pathogenesis of HMS.

**Pathogenesis of HMS**

A summary of the pathogenetic pathways is shown in figure 20. In Nigeria spleens reach their maximum weight at 15-19 years of age and begin shrinking at 50 years. In the Upper Watut Valley in Papua New Guinea where there is a very high incidence of HMS, gross splenomegaly takes 5-10 years to develop [Crane 1985] and so the syndrome is very unusual in children under the age of 10. HMS can occur at any age after childhood the peak incidence being in the third and fourth decades, and is more common in women than men. Individuals with HMS seem to generate active malarial immunity at the normal rate because the frequency and severity of parasitaemias decreases with age, but this is not accompanied by the normally expected splenic regression [Crane 1981].

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Figure 20.

Suggested pathogenesis of HMS

Cytotoxic antibodies
↓
Reduced T suppressor cells
↓
Malaria → Polyclonal increase in B lymphocytes
↓
High IgM levels
↓
Immune complexes
↓
Splenomegaly and anaemia
The role of malaria

It is unusual to find malaria parasites in the blood of HMS patients [Hoffman 1984], and they may even have fewer parasitaemic episodes than normal local controls [Crane 1981]. As a result of B lymphocyte stimulation by repeated malaria infection they have high anti-malarial antibody titres. All human types of malaria parasite have been implicated, but there is some geographical variation in the most common type associated with HMS.

Distribution of haemoglobin genotypes in patients with splenomegaly

Indirect evidence for the involvement of malaria in HMS is provided in this study by the under-representation of AS genotype in patients with HMS and those in group D, 12/19 of whom had a good response to proguanil. A lack of sickle trait genotype in HMS has also been described in patients from Zambia and Nigeria [Lowenthal 1980, Bryceson 1976, Hamilton 1969, Watson-Williams and Allan 1968]. Haemoglobin AS is associated with a partial resistance to *Plasmodium falciparum*. Parasitised cells containing haemoglobin AS sickle more easily than non-infected cells due to a reduced intracellular pH. These damaged cells are preferentially phagocytosed by monocytes and this may explain why these patients are relatively resistant to severe falciparum infections.
Genetic factors

Repeated malaria infection cannot be the sole reason for the development of HMS since only a few people living in the same area develop the disease. It is likely that host factors, particularly genetic predisposition, are important as the syndrome is known to have a predilection for certain families and tribal groups. In Zaria, Northern Nigeria, 50% of patients with HMS admitted to a hospital were Fulanis, whereas this tribal group only made up 3% of other hospital patients. [Bryceson 1976]. Familial aggregation of cases has also been noted in Uganda [Ziegler 1972] and Papua New Guinea [Crane 1981].

The genes of the human major histocompatibility complex (MHC) have been investigated in HMS and associations with both class 1 and class 2 antigens have been described. In Tanzania, certain HLA A and B locus antigens are associated with very high titres of antibodies to Plasmodium falciparum [Osoba 1979] while in Papua New Guinea, HLA DR2 is associated more with massive splenomegaly than moderate splenomegaly [Bhatia 1985].

Jensen [1984] compared the nature of the immune response to malaria in southern Sudan, where splenomegaly is rare, and Flores, Indonesia where large spleens are common. The prevalence of Plasmodium falciparum was similar in both areas.
and yet the Sudanese sera contained a factor which could retard the intra-erythrocytic parasite development and the Indonesian sera did not. This factor was capable of rendering the parasites moribund thereby reducing the production of invasive merozoites. Rodent models have shown that these moribund parasites are able to activate cell-mediated immune mechanisms. In contrast, the Indonesian sera possessed a merozoite-invasion-blocking antibody in much higher titres than Sudanese sera. The authors postulated that the higher incidence of splenomegaly in Indonesia was the result of continuous antigenic stimulation by active merozoites whereas in Sudan where splenomegaly was rare, reduced growth and production of parasites resulted in lower antibody levels. As the spleen is an important site of clearance of immune complexes greater splenic enlargement would occur in the patient group with the higher antibody production. The authors concluded that these observations might be explained by genetic differences in the availability of T cell subsets concerned with cell-mediated immunity.

An abnormal functional immune response is probably not responsible for the high immunoglobulin levels in HMS. Sera from both HMS and non-HMS Indonesian patients living in a holoendemic area showed no difference in their ability to inhibit in vitro growth of *Plasmodium falciparum* [Campbell 1986]. This was measured by the uptake of $^3$H hypoxanthine into new parasite DNA and inhibition occurred prior to merozoite reinvasion.
**Antibody production and immune complexes**

I have found very high levels of IgM in both SLVL and HMS patients in Ghana (figures 18 and 19). This may be due to chronic exposure to malaria or genetic predisposition. Population studies have shown that in HMS high IgM levels precede the splenomegaly by some years and therefore represent an earlier stage in the evolution of the disease [Crane 1971]. High molecular weight immune complexes consisting mainly of IgM, circulate in the serum of HMS patients for prolonged periods in the absence of recurrent parasitaemic episodes and may be demonstrated in Kuppfer cells [Crane 1986b]. The consequent expansion of the reticuloendothelial system could account for both the splenomegaly and the hepatomegaly which invariably occurs in the disorder. The levels of IgM, but not IgG, are proportional to the splenic size [Ziegler 1973] and can be reduced by anti-malarial drugs. The IgM level in HMS parallels the specific anti-malarial antibody titre [DeCock 1986], most of the IgM is polyclonal and directed against altered IgG not against malarial antigens. The overproduction of IgM is thought to be due to a lack of immunoregulatory control over IgM production by B cells.

**Abnormalities of lymphocyte subsets**

Although this research has shown that SLVL in Ghana is an
expansion of B-lymphocytes, the relative proportions of T-lymphocyte sub-sets and the CD4:CD8 ratio in SLVL and non-SLVL patients were similar to local controls. HMS patients from Africa but not elsewhere [Pryor 1967] may have expansion of B-lymphocytes outside the liver and spleen [Watson-Williams and Allan 1968, Fakunle 1978]. Data from Indonesia [Hoffman 1984], Papua New Guinea [Crane 1986b] and Nigeria [Fakunle 1978] show that there is also a relative or absolute circulating T cell lymphopenia. Studies from Indonesia have demonstrated that this is due to a specific reduction in T8+ suppressor/cytotoxic lymphocytes with a T4+:T8+ ratio of 1.93:1 in HMS compared to a local control population who had ratios of 1.5:1 or less [Hoffman 1984]. An IgM antibody has been detected in the serum of HMS patients which was cytotoxic for T8+ cells from normal donors [Piessens 1985]. Like other parameters in HMS, the T4+:T8+ ratio returns to normal after treatment with anti-malarial drugs. Similar quantitative or qualitative T cell defects occur in systemic lupus erythematosus [Morimoto 1979] and leprosy [Bullock 1982], and the resultant lack of suppressant activity facilitates the continued polyclonal activation of B lymphocytes.

**AN ANALOGY BETWEEN SLVL AND BURKITT'S LYMPHOMA?**

This study confirms that a progressive reduction in splenic size in response to anti-malarial therapy defines a group of
patients who have polyclonal lymphoproliferation despite some individuals having blood findings suggestive of SLVL. Complete failure of this treatment only occurs in SLVL patients with evidence of monoclonality. There is an intermediate group of patients with partial or transient responses to proguanil and high levels of villous lymphocytes in whom the lymphoproliferation may be mono- or polyclonal. These findings are not unexpected if HMS evolves into a clonal malignant disease in some individuals. Alternatively, there may be two unlinked populations of cells in the same individual; one induced by malaria and therefore polyclonal and sensitive to proguanil and another, de-novo spontaneous monoclonal lymphocyte expansion. This seems unlikely and there is no evidence that proguanil has any anti-tumour activity in such malignant diseases.

It is possible that in HMS the polyclonal expansion of lymphocytes provides targets for somatic mutation and that there is eventually selection of a single clone whose growth advantage leads to the development of SLVL. The moderate response to proguanil may be due to the temporary co-existence of the malignant clone and the polyclonal expansion from which it arose. An alternative explanation is that the malignant clone could still be partially antigen-dependent and an antigen-independent sub-clone is rapidly selected by proguanil therapy. The polyclonal cases with moderate response may have a different origin. However, a transformed, or partially transformed polyclonal proliferation is possible with viral
aetiology. These cases would then be expected to evolve by clonal selection into a clonal disease.

This process would be analogous to that leading to endemic Burkitt's lymphoma in which polyclonal lymphoid expansion due to malaria is also thought to be part of a multi-step process [Sugden 1989]. Malaria induces an increased population of B cells in which oncogenic events could occur (figure 21). Epstein-Barr virus can immortalise B cells and the presumed key event in the progression from polyclonal B cell expansion to monoclonal Burkitt's lymphoma is a chromosomal translocation. This juxtaposes the MYC proto-oncogene next to a rearranged immunoglobulin gene locus which leads to deregulation of the gene and eventual selection of a single clone. Although HMS occurs in the same areas as Burkitt's lymphoma it is much less aggressive so the two diseases probably involve different co-factors. The transformation of HMS is likely to involve a much weaker oncogenic sequence than MYC such as BCL-1 or BCL-2 both of which have been associated with B cell lymphomas [Griesser et al 1989].
Figure 21. LIKELY EVENTS IN PATHOGENESIS OF ENDEMIC BURKITT'S LYMPHOMA

- Immortalised cell
- EBV
- Lymphocyte
- Malaria
- Polyclonal cells
- C-MYC translocation
- Burkitt's lymphoma
I did not demonstrate any rearrangement of the BCL-1 locus in 3 patients with SLVL in Ghana. However, only one probe was used for the major translocation cluster and other breakpoint sites have been described which were not screened in this study. Translocations involving the BCL-1 proto-oncogene, t(11:14), have been described in pro-lymphocytic leukaemia, CLL and diffuse small and large cell non-Hodgkin's lymphomas and more recently in SLVL [personal communication, Dr D Jadayell]. 3/18 Caucasian SLVL patients have cytogenetic breakpoints on chromosome 11q13 not all of which occur within the major translocation cluster of the BCL-1 gene; some are located telomeric to this region. The actual site of translocation on the immunoglobulin gene in SLVL has not yet been established but lies within the heavy chain gene. PRAD 1 gene mRNA of 4.4kb, or a truncated version of 1.35kb, has only been detected in SLVL cases involving the BCL-1 locus. It therefore appears as if there is an interaction between the immunoglobulin gene enhancer and the PRAD 1 promoter although this would have to be over some distance as most breakpoints occur 70-100kb away from the PRAD 1 locus. Alternatively, the break in the gene may remove an inhibitory effect so that the PRAD 1 gene is overexpressed in translocations involving the BCL-1 locus.

**IMPLICATIONS FOR FUTURE RESEARCH**

Before the completion of this study, in order to establish a relationship between HMS and SLVL it would have been necessary
to follow a large cohort of HMS patients for many years clinically, morphologically and at a molecular level to obtain evidence of evolution of a monoclonal population of cells. However, this study has identified a sub-group of patients, some of whom have HMS, who also have an expansion of their circulating villous lymphocyte pool without evidence of a monoclonal process. A careful follow-up of these patients with regular assessment of lymphocyte clonality could considerably shorten the time of observation required to detect the evolution of a malignant population of cells.

In order to increase the detection rate this approach could be combined with more sensitive methods, such as polymerase chain reaction (PCR), to screen patients for the presence of small populations of clonal, malignant cells. In theory PCR could detect a single target molecule of DNA in a complex mixture of other DNA providing the sequence of the DNA flanking the area of interest was known. However, the procedure is complicated when applied to immunoglobulin genes because the normal process of gene rearrangement means that the flanking sequences are not constant.

The factors involved in the multi-step lymphomagenesis of SLVL are not known although it appears as if HMS, and hence malaria, may be implicated. There is circumstantial evidence that Epstein-Barr virus plays a role in the evolution of Burkitt's lymphoma. By analogy it is also possible that there is viral involvement in the genesis of SLVL in Ghana.
Epidemiological studies of the distribution of oncogenic viruses in areas where both HMS and SLVL are common would be informative. At a molecular level DNA analysis could be used to screen cases of HMS and SLVL for viral genetic material which has been integrated into the cellular genome. Oncogenic viruses known to be present in the Ghanaian population include HIV-1, HIV-2, HTLV-1, HTLV-2 and Epstein-Barr. Oncogene rearrangements themselves, particularly BCL-1, need to be searched for in a larger number of Ghanaian patients with HMS and SLVL using probes directed at several different regions of the gene which are known to be involved in other B-cell malignancies.

CONCLUSION

This thesis describes a sub-type of non-Hodgkin's lymphoma, splenic lymphoma with villous lymphocytes, for the first time from West Africa. Its clinical, serological, immunological and molecular features are characterised. The implications of its existence are discussed with respect to the management of splenomegaly and the analysis of cofactors involved in lymphomagenesis in tropical Africa.
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APPENDICES
SPLENIC LYMPHOMA WITH VILLOUS LYMPHOCYTES IN GHANA

(accepted for publication in September issue of The Lancet)


St George's Hospital Medical School, London
*Komfo Anokye Teaching Hospital, Kumasi, Ghana
Summary

Ten cases of Splenic Lymphoma with Villous Lymphocytes (B-SLVL) from Ghana are discussed. This disorder, not previously reported from Africa, is a monoclonal B lymphoproliferative disease characterised by splenomegaly and distinctive villous lymphocytes in the peripheral blood. SLVL seems to be more common in Ghana than in temperate regions although the clinical presentation is similar. In Africa the lymphocyte count is higher than in Europe and the disorder affects middle-aged women rather than elderly men. It is likely that formerly, SLVL has been categorised as 'splenic' chronic lymphocytic leukaemia or Hyper-reactive Malarial Splenomegaly.
The characterisation of lymphoma in Africa is often difficult due to lack of technical resources, so there is a dearth of information concerning non-Burkitt’s, non-Hodgkin’s lymphomas. The incidence of these sub-types in those aged 20-50 is greater than in temperate climates but there is little data on the histology. In general, follicular histology is rare and the lymphoid tumours tend to be more aggressive in Africa.

In temperate zones splenic lymphoma with villous lymphocytes (SLVL) is predominantly a disease of elderly males, which presents with splenomegaly, tiredness and malaise. Paraproteins are found in serum or urine in over 50% of European patients. In the absence of lymphocyte phenotypic data, SLVL may be diagnosed in patients with a peripheral blood lymphocyte count >10 x 10^9/l when at least 30% of these cells have a typical “villous” appearance. Villous lymphocytes are larger than those of chronic lymphocytic leukaemia (CLL) with a clumped nuclear chromatin pattern and often a nucleolus. The cytoplasm is basophilic with characteristic short cytoplasmic villi which, unlike those in hairy cell leukaemia, are unevenly distributed at one or both poles of the cell. The cellular phenotype differs from hairy cell leukaemia usually lacking CD25, CD11c and tartrate-resistant acid phosphatase markers. If the membrane markers are suggestive, SLVL may be diagnosed in patients with lymphocyte counts <10 x 10^9/l.

We noted that the lymphocyte morphology in Ghanaian cases of...
clonal lymphoproliferative disease associated with, and possibly arising from, hyper-reactive Malarial Splenomegaly (HMS) resembled that of SLVL. We therefore sought morphological, immunophenotypic and molecular evidence for B-SLVL in further patients from the Ashanti region.
Methods

Patients
Between 1986 and 1990 10 Ghanaian patients attending the haematology clinic in Kumasi, Ghana were selected for study on the basis of a peripheral blood lymphocyte count >10 \times 10^9/\text{l} when at least 30% of these cells demonstrated characteristic villous morphology. All patients gave informed consent and the study was approved by the National Ethical Committee of Ghana.

Techniques
Serum electrophoresis was carried out using cellulose acetate paper with a continuous tris-EDTA-glycine (pH9.3) buffer system. Identification of paraproteins detected was performed by the protein laboratory at Queen Mary's Hospital, Roehampton.

Peripheral blood slides for morphology and surface marker studies were prepared in Ghana, air-dried, wrapped unfixed in aluminium foil and frozen prior to transportation to the UK. Following thawing and fixing, the blood films were exposed to a panel of monoclonal antibodies to lymphocyte surface antigens and membrane-associated immunoglobulin light chains using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. Cell morphology was ascertained on Wright's stained smears.

Samples of DNA were obtained in Ghana by a simple method appropriate for tropical laboratories with limited facilities. In the UK, DNA was digested with restriction enzymes, size fractionated in agarose gels and Southern blotted onto nylon
membranes prior to hybridisation to a $^{32}$P-labelled DNA probe for the immunoglobulin heavy chain locus. Gene rearrangements were detected by autoradiography of the membranes. The blots were sequentially hybridised to probes for TCR-gamma (J1), bcl-1 (major translocation cluster) and bcl-2 (major breakpoint cluster) loci.
Results

Clinical features

The 10 patients, 3 men and 7 women aged 35-66 years (mean 53 years), presented with splenic discomfort (9), anorexia and weight loss (8), recurrent fevers (5), cough (2) and headaches (1). Splenomegaly was universally present varying from 8-41cm (mean 21cm). Hepatomegaly (3-15cm) and anaemia (mean haemoglobin 8.5g/l) were common (9/10); 1 patient had generalised lymphadenopathy. Of the 7 patients available for follow-up, two died from infections within 1 year and 1 died from acute haemolysis. 2 patients are alive but unwell (pyomyositis/persistent diarrhoea) 8 and 10 months after presentation and the remaining 3 patients are well.

Lymphocyte characteristics

The peripheral blood lymphocyte counts varied from 13-75 x 10^9/l (mean 36) villous lymphocytes comprising 34-87% (mean 66%) (figure 1). Lymphocyte phenotype studies in 5 patients revealed that the majority of cells were CD5-, CD11c- B lymphocytes (CD19+ 79-96%). Surface expression of immunoglobulin light chains was restricted to lambda in 3 patients and to kappa in one. Bone marrow aspirates from 3 patients showed infiltration with 75-86% lymphocytes.

Serological and molecular results

Two small paraprotein bands characterised by immunofixation as IgMk and IgGk were detected in the serum of 1/5 patient. All cases tested (5/5) had rearrangements of their immunoglobulin
heavy chain gene. No abnormalities were detected on probing for rearrangements of the T-cell gamma receptor (0/4), bcl-1 (0/3) or bcl-2 (0/4).
Discussion

These 10 Ghanaian patients fulfill the criteria for the diagnosis of B-SLVL. In western countries B-SLVL is a rare disorder: it appears to be more common in Ghana. These 10 patients were seen at a general hospital by one doctor over a period of 4 years.

In equatorial Africa SLVL is similar to the disorder in temperate climates. It is a monoclonal B-lymphoproliferative disorder with characteristic morphological features and surface markers. In both areas splenic discomfort and anorexia are the most frequent symptoms, hepatosplenomegaly is common and involvement of the bone marrow by villous lymphocytes is usual. The course of the untreated disease in Africa varies from indolent, with little or no clinical deterioration over 1-2 years, to a debilitating illness culminating in death from infection within weeks of diagnosis.

The frequency of oncogene rearrangements in SLVL is unknown. The oncogenes investigated in this study are known to be associated with B-cell neoplasms, bcl-1 particularly with centrocytic lymphoma and bcl-2 with follicular lymphoma. We used probes for the major breakpoint region of each oncogene. There are other breakpoint sites for both bcl-1 and bcl-2 which were not investigated so the failure to detect abnormalities in these individuals does not exclude the possibility of rearrangements elsewhere in the genes.

In Africa, where facilities for molecular and phenotypic studies
are limited, it is likely that cases of SLVL have been overlooked. A study of CLL among Congolese Bantus, for example, recognised two distinct groups\(^3\). One was indistinguishable from classical European and North America CLL and the other was associated with massive hepatosplenomegaly, a less pronounced lymphocytosis (4-50 x 10\(^9\)/l) and high gamma globulin with monoclonal bands. Although no morphological data was given these findings would be consistent with SLVL.

Specific diagnosis of SLVL has therapeutic implications: removal of the spleen early in the disease may be beneficial\(^10\). Splenectomy, unlike many forms of anti-cancer therapy, is available and affordable in the tropics but should not be undertaken in malarious zones without definite indications.

The comparatively high frequency of SLVL in a malaria hyperendemic region of Ghana is of interest. HMS has features in common with African SLVL such as female predominance, splenic enlargement and lymphocytosis. The association of HMS and SLVL is supported by a review of the lymphocyte morphology in 4/5 patients reported by us with HMS, CLL or clinical overlap syndromes\(^4\). All had immunoglobulin gene rearrangements and 2 had villous lymphocytes exceeding 30% thus establishing a diagnosis of B-SLVL. Retrospective analyses of lymphocyte morphology from African patients previously thought to have CLL may yield further cases of SLVL.
Only a careful long-term study of a large number of patients with HMS will provide an answer to the intriguing question of the malignant potential of HMS. If a link between HMS and SLVL is confirmed, the role of other aetiological factors, particularly viruses, in lymphomagenesis can be investigated.

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8. Annotation
The role of the bcl-2 gene in lymphoma

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Haematologica 1986; 71: 25-33
Use of immunoglobulin gene rearrangements to show clonal lymphoproliferation in hyper-reactive malarial splenomegaly

IMELDA BATES  GEORGE BEDU-ADDO  DAVID H. BEVAN  TIM R. RUTHERFORD

In Africa, hyper-reactive malarial splenomegaly (HMS), which is also known as tropical splenomegaly syndrome, can be associated with a prominent lymphocytosis in blood and bone marrow that is difficult to distinguish clinically from chronic lymphocytic leukaemia (CLL). The observation that some patients with HMS become resistant to treatment with anti-malarial drugs has led to the suggestion that HMS may evolve into a malignant lymphoproliferative disorder. To test this hypothesis, 22 Ghanaian patients with HMS and/or lymphocytosis were categorised by degree of response to proguanil according to standard clinical criteria, and DNA was extracted from peripheral blood cells and screened for rearrangements of the Jh region of the immunoglobulin gene with a DNA probe. Clonal rearrangements of the Jh region were found in all 3 patients with no response, in none of 13 patients with sustained response, and in 2 of 6 patients with moderate response or relapse on proguanil therapy. The detection of such rearrangements, and hence clonal lymphoproliferation in individuals with clinical features intermediate between HMS and CLL, supports the hypothesis that HMS may evolve into a malignant lymphoproliferative disorder.


Introduction

Hyper-reactive malarial splenomegaly (HMS) occurs in most countries in the malaria belt and is characterised by massive splenomegaly, which diminishes with antimalarial treatment. The pathophysiology of HMS is poorly understood; a disordered immune response to malarial parasites leads to overproduction of B lymphocytes and immune complexes, with consequent reticuloendothelial hypertrophy. The excess production of B cells may be enhanced by cytotoxic antibodies (of uncertain relation to malaria infection) against suppressor T cells.

Diagnosis of HMS is based on the exclusion of other locally prevalent causes of massive splenomegaly and regression of the spleen on anti-malarial therapy. Hepatic sinusoidal lymphocytosis together with IgM levels that are significantly higher than the local mean corroborate the diagnosis.

In about 10% of individuals in Africa, the disease resembles chronic lymphocytic leukaemia (CLL)—ie, HMS is associated with increased numbers of lymphocytes in blood and bone marrow. This overlap led to the suggestion that HMS might be a pre-malignant state that could evolve into CLL.

The usual presentation of CLL in Africa (with splenomegaly rather than with lymphadenopathy) and lack of local facilities for lymphocyte phenotyping mean that the placing of patients with splenomegaly in HMS, CLL, or overlap categories is dependent on lymphocyte counts and response to proguanil. Current criteria for the diagnosis of CLL (peripheral blood lymphocyte count > 10 x 10^9/l and bone-marrow lymphocytes > 30%) were derived from European and North American populations. Because it is not clear whether these criteria can discriminate between
IMMUNOGLOBULIN GENE REARRANGEMENTS AND RESPONSES TO PROGUANIL

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PBLC = peripheral blood lymphocyte count (× 10⁹/l); BMX = percentage of bone marrow lymphocytes; Ig M = immunoglobulin gene rearrangements; Resp = response to proguanil: + = good; +/− = moderate; − = none. ND = not done.

Residual proteins were precipitated by the addition of saturated sodium chloride. Ethanol was added to precipitate DNA from the supernatant; DNA was then recovered by spooling. Latterly, DNA was prepared from fresh blood samples by a second method. Briefly, whole blood samples were lysed with "Triton-X 100", and the nuclei were pelleted by centrifugation and lysed in guanidinium thiocyanate buffer. This process rendered the samples non-infective before transport to the UK, where DNA was further purified by isopycnic banding in cesium chloride.

DNA was digested with restriction enzymes in the manufacturer's recommended buffer after addition of 1-4 units of sparnexidine. Digestion of DNA was subjected to electrophoresis through 0-8% agarose gels, Southern blotted onto nylon membranes, and hybridised to a nick-translated, ³²P-labelled DNA probe for the Jh region of the immunoglobulin gene. All rearrangements detected in HMS patients after autoradiography of the membranes were confirmed by digestion of the native DNA with a second restriction enzyme before Southern blotting and reprobing.

Results

Gene rearrangements (table)

Rearrangements of the immunoglobulin heavy chain gene were not detected in the 3 negative control patients with chronic myelogenous leukaemia but were demonstrated in all 3 patients (18, 21, 22) with C.LL. All 13 patients in group A showed a normal germline pattern with no rearranged bands.

2 of the 6 patients in group B had rearrangements of the immunoglobulin gene Jh region: patient 18 had blood and bone marrow findings that would classify him as C.LL by current criteria and a 20% reduction in splenic size after antimalarial treatment; and the spleen of patient 16 reduced by only 27% despite more than 6 months of treatment. None of the other 4 patients in this group had any rearrangements. They had decreases in splenic size from 32 to 62%, which were not sustained; their spleens enlarged again after 3-12 months despite treatment.
All 3 patients in group C had rearrangements of the immunoglobulin gene heavy chain. Patient 20 received proguanil for more than 12 months without any reduction in splenic size. The cost of a trial of proguanil could not be justified in patients 21 and 22 who, as judged by their lymph node and liver size, had C.L.L.

Patients 20 and 16 were of especial interest since they had PBLCs of less than $15 \times 10^9/l$, making C.L.L. unlikely, and yet also had immunoglobulin gene rearrangements. By contrast, patient 18, who had a PBLC compatible with a diagnosis of C.L.L., had a moderate response to proguanil.

Patient 20—This 58-year-old woman presented with a spleen measuring 41 cm, which increased to 44 cm despite more than 12 months of treatment with proguanil. Her IgM was 2.5 g/l (local normal mean = 1.9 g/l; unpublished). Her PBLC increased steadily during this time to $27 \times 10^9/l$ and she died after a severe haemolytic episode—a common complication of H.M.S.

Patient 16—The spleen of this 40-year-old woman measured 11 cm at presentation and reduced to 8 cm after 4 months of treatment before she was lost to follow-up.

Patient 18—This patient presented with an enlarging spleen and was treated with daily proguanil for 3.5 months before he was lost to follow-up. During this time his spleen reduced from 10 cm to 7 cm and his liver also reduced from 7 cm to 5 cm. His IgM was raised at 2.8 g/l.

Age and response to proguanil

Patients with a good response to proguanil were significantly younger than those in either of the other two groups ($p = 0.015$); however, we realise that a larger patient sample should be assessed. There was also a wide variation in the numbers of lymphocytes in the peripheral blood and bone marrow of patients in group A; the PBLC varied from 1 to 11 $6 \times 10^9/l$ and the BM% from 12 to 44%. Even in group B, the PBLC varied from 0.7 to 5.7 $10^9/l$ and from 19 to 66%, respectively (excluding patient 18 who had C.L.L.).

Discussion

Our findings confirm that progressive splenic response to proguanil defines a group of patients with H.M.S. who have polyclonal lymphoproliferation despite blood and bone marrow findings suggestive of C.L.L. We have also found that complete failure of proguanil treatment usually occurs in monoclonal lymphoproliferative disease, which might not be obvious depending on the cell count. There is also a heterogeneous category of H.M.S. patients with transient responses to proguanil in whom lymphoproliferation may be either polyclonal or monoclonal, irrespective of blood count. This finding is what would be expected if polyclonal H.M.S. was evolving into a clonal C.L.L-like malignant disease in some individuals. Alternatively, there may be unlinked polyclonal (malaria-induced, proguanil-sensitive) and de novo monoclonal (spontaneous) lymphocyte expansions in the same individual while under observation, but this seems unlikely. There is no evidence that proguanil has any anti-tumour activity in spontaneous lymphoproliferative malignant disease.

We prefer the hypothesis that in H.M.S. the dysregulated polyclonal expansion of lymphocytes provides targets for somatic mutation and that there is eventually selection of a single clone whose growth advantage leads to the development of C.L.L. The moderate response to proguanil may be due to the temporary coexistence of the malignant clone and the polyclonal expansion from which it derived. An alternative explanation is that although the malignant clone could still be partly antigen dependent, an antigen-independent subclone is rapidly selected by proguanil therapy. The polyclonal cases with a moderate response may have a different origin. However, a transformed or partly transformed polyclonal proliferation is possible with a viral aetiology. These cases would then be expected to evolve by clonal selection into a clonal disease. This process would be analogous to that leading to endemic Burkitt's lymphoma, in which polyclonal lymphoid expansion due to malaria is also thought to be part of a multistep process. Malaria induces an increased population of B cells in which oncogenic events may occur. Epstein-Barr virus can immortalise B cells, and the presumed key event in progression from polyclonal B-cell expansion to monoclonal Burkitt's lymphoma is a chromosomal translocation. This juxtaposes the MYC proto-oncogene to a rearranged immunoglobulin gene locus, which leads to deregulation of the gene and eventual selection of a single clone. Although H.M.S. occurs in the same areas as does Burkitt's lymphoma, it is a much lower grade malignant disease, so the two diseases probably involve different cofactors. The aggressive nature of Burkitt's lymphoma may be due to the strongly oncogenic character of the MYC gene. The transformation of H.M.S. is likely to involve a weaker oncogenic sequence, such as BCL-1 or BCL-2, both of which have been associated with C.L.L. and follicular lymphoma.

We thank Dr. T. Rabbits for the gift of probes and Professor Gordon-Smith for his helpful comments. I.B. is a Wellcome Research Fellow and T.R. is a Wellcome University Award Lecturer.

REFERENCES

Extracting, storing, and transporting whole blood DNA under tropical conditions

I Bates, G Bedu-Addo, T R Rutherford

Abstract
A simple and robust technique for the extraction of DNA under tropical field conditions is described. It requires minimal equipment and is based on lysing cells in whole blood and precipitating the nuclei containing the DNA by centrifugation. The DNA solution can be stored in guanidinium buffer for many months without being refrigerated. Further purification of the DNA can then be carried out in a laboratory with facilities for ultracentrifugation by banding the DNA through cesium chloride. This method yields DNA of sufficient quality and purity for Southern blotting and probing and alleviates the need to transport whole blood between different countries and laboratories.

The application of DNA analysis to the study of tropical diseases is difficult because of problems with blood transportation and storage, or DNA extraction under field conditions. We have developed a simple, reliable method of extracting DNA from whole blood which can be carried out in a small hospital laboratory. DNA is obtained in a form suitable for storage without refrigeration at ambient tropical temperatures for many months.

Methods and results
At least 5 ml anticoagulated blood are mixed with 50 ml cell lysis buffer (figure). Nuclei are isolated by centrifugation at 2500 rpm for 10 minutes and the supernatant discarded. The nuclear pellet is vigorously resuspended in the last drop of liquid and mixed with 1-2 ml nuclear lysis buffer (figure). The DNA solution is transferred to a 2 ml tube and can be stored in this form for many months at ambient temperatures before purification in the United Kingdom.

Further purification is achieved by layering the nuclear lysate over a 3 ml pad of 1 g/ml cesium chloride in 0.1 M EDTA and centrifuging at 32,000 rpm for 16 hours. The DNA band is removed with a wide bore pipette and extensively dialysed against 10 mM TRIS 1 mM EDTA (pH 7.5) (1 x TE) to remove traces of guanidinium and cesium chloride.

The level of degradation of the DNA was assessed by electrophoresing 0.5 µg DNA through an agarose gel and observing the size of the DNA after staining with ethidium bromide. In most samples only DNA of large molecular weight is present. A few samples contain small amounts of low molecular weight DNA, shown by smearing on the gel, and are therefore slightly degraded. Nevertheless, even in samples which exhibit some degradation the DNA is sufficiently pure to permit endonuclease digestion with enzymes, such as Hind III, Bam HI, providing 1-4 mM spermidine are added to the manufacturer’s recommended buffers. The samples can be Southern blotted and reproducible restriction fragments are seen for each sample when probed for the heavy chain region of the immunoglobulin gene.

Constituents of buffers

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<td>Cell lysis buffer</td>
<td>3 ml 1M TRIS (pH 7.5) 32.9 g sucrose 1.5 ml 1M magnesium chloride 3 ml Triton X-100 Water to 300 ml</td>
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<tr>
<td>Nuclear lysis buffer</td>
<td>50 g guanidinium thiocyanate 0.5 g sodium-N-laurylsarcosinate 2.5 ml 1M sodium citrate (pH 7.0) 0.7 ml 2-mercaptoethanol Water to 100 ml</td>
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We have used this method to extract and purify DNA from 22 20 ml blood samples from Ghanaian patients with a variety of haematological disorders including chronic leukaemias. An average of 680 µg DNA was obtained from each sample (range 13-324 µg), the largest yields coming from leukaemic patients. Eighty per cent of samples yielded over 30 µg DNA, and even in samples containing less DNA, there was adequate material for 1-3 Southern blots. Specimens took 1.5-8.5 months (average 3.8) to reach our United Kingdom laboratory during which time they
were not refrigerated but stored and transported at the ambient Ghanaian temperature.

An advantage of this method is that it alleviates the need to transport whole blood between laboratories. This method almost certainly renders the samples non-infective for lipid-enveloped viruses such as HIV and hepatitis B, because guanidinium thiocyanate is a powerful protein denaturant used for extracting RNA from tissues rich in ribonucleases. Triton X-100 is also a potent non-ionic surfactant which, in combination with a solvent, has been shown to produce more than 10^5-fold reduction in HIV-1 infectivity.

These substances disrupt the viral membranes, essentially dissociating the viral genome from its receptor, making it incapable of infecting host cells.

This work was supported by the Wellcome Trust.


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Use of leucocyte alkaline phosphatase (LAP) score in differentiating malignant from benign paraproteinaemias

G Majumdar, M Hunt, A K Singh

Abstract

The leucocyte alkaline phosphatase (LAP) score of peripheral blood neutrophils was examined in 20 patients with multiple myeloma and compared with the score in 18 patients with monoclonal gammopathy of undetermined significance (MGUS). The mean (95% confidence limit) LAP score in those with multiple myeloma was 186 (169-218) compared with 92 (64-120) in the MGUS group. In the multiple myeloma group, all but one patient had a high LAP score, irrespective of disease. No cause for raised LAP, such as infection, was present in any of the patients with multiple myeloma. In the MGUS group six patients had a raised LAP score; in two of them another cause for such a rise was present (autoimmune haemolytic anaemia and primary thrombocytopenia). In neither group did the LAP score correlate with duration of the disease, bone marrow plasma cell count, paraprotein concentration, haemoglobin, total white cell or neutrophil count.

It is concluded that a normal LAP count in patients with paraproteinaemia suggests a benign condition, but a raised count does not indicate a malignant condition.

Monoclonal gammopathy is a common disorder, especially in the elderly, but only a small percentage of these patients have overt multiple myeloma or related conditions at the time of diagnosis. The rest are diagnosed as having MGUS and usually followed up for an indefinite period as a significant proportion of them progress to multiple myeloma and related malignant disorders. Several tests have been proposed for differentiating malignant from benign paraproteinaemia but none has been found to be fully reliable. The LAP score has been recommended as a useful test for such differentiation. We examined the LAP score in patients with multiple myeloma and MGUS to assess the value of this test in differentiating malignant from benign paraproteinaemias.

Methods

Twenty patients (11 men, nine women, mean age 67 years) with multiple myeloma diagnosed by the standard criteria were included in this study. Four were newly diagnosed, 11 were in the plateau phase and five were in relapse. The MGUS group comprised 18 patients (10 men, eight women, mean age 63 years) with paraproteinaemia who did not fulfil the diagnostic criteria for multiple myeloma and were followed up for at least 24 months without my change in disease course. None in either group had a raised white cell count at the time of the present investigation. Blood films were made at the time of routine follow-up and were stained for LAP by using a commercial kit (Diagnostica Merck). Scoring
MASSIVE SPLENOMEGALY IN THE TROPICS
AND ELECTIVE SPLENECTOMY

(accepted for publication in September issue of Surgery)

I Bates

Wellcome Tropical Research Fellow
St George's Hospital Medical School, London
Massive splenomegaly in the tropics and elective splenectomy

Many non-urgent splenectomies are carried out misguided due to ignorance concerning the causes and management of gross splenic enlargement. Surgeons find themselves pressurised by patients who are convinced that if their spleens are removed they will be 'cured'. Such surgery is fraught with difficulties. The operative mortality and the risk of overwhelming post-operative sepsis in tropical areas means that elective splenectomy should only be considered as a last resort.

Prevalence

The prevalence of splenomegaly in tropical climates varies between different geographical regions. An editorial states that 'in many tropical countries, all spleens are big; some are bigger than others'. For example, the average adult spleen in Nigeria weighs 271g, 100g more than in Europeans. In certain villages in Papua New Guinea, 80% of people have palpable spleens which are almost exclusively due to the tropical splenomegaly syndrome.
The diseases which cause splenomegaly in temperate countries are also present in tropical areas but in addition, the warm climate and poor sanitation facilitate infection with bacteria and parasites. Stimulation of phagocytosis and antibody formation then results in reticulo-endothelial hypertrophy and splenic enlargement.

**Definition**

'Massive' spleens are generally accepted as measuring 10cm or more from the left costal margin to their tip in the anterior axillary line. The measurement should be made with the patient lying flat, carefully following the contour of the spleen to account for expansion into the left flank (figures 1 and 2).

**Causes of massive splenomegaly**

In the tropics these include infectious and haematological disorders (figure 3). Cirrhosis, splenic abscesses, brucellosis, tuberculosis and haemoglobinopathies may also occasionally produce marked splenic enlargement.
In patients with massive spleens a diagnosis can usually be made without difficulty on the basis of the clinical features and investigations and with a knowledge of locally prevalent infections. Confusion may arise when a diagnosis cannot be reached and patients are mistakenly labelled as having tropical splenomegaly syndrome (TSS).

Tropical Splenomegaly Syndrome

This is a specific disorder and cannot be used as an 'umbrella' diagnosis for all cases of gross splenic enlargement with no obvious cause. It has a worldwide, but patchy, distribution within the malaria belt.

Pathogenesis (figure 4)
TSS is a failure of splenic regression after normal development of immunity to malaria. It is rare in children under 10 years because this immunity takes 5-10 years to develop. The basic defect appears to be a lack of T suppressor cells responsible for modulating B cell activity. There is excessive antibody production, especially of IgM, in response to repeated malaria infections. Large amounts of circulating immune complexes containing IgM are formed and result in splenomegaly. The complexes are
also deposited in the Kupffer cells in the liver where they are associated with a reactive sinusoidal lymphocytosis. It has been proposed that TSS be renamed 'hyper-reactive malarial splenomegaly' to reflect the important role played by malaria in its aetiology and to stress its importance as a specific clinical entity.

Clinical and laboratory features
TSS occurs most commonly in women under 30 years although either sex may be affected. Patients are usually surprisingly well and may have had splenomegaly for many years without any symptoms. Abdominal discomfort or tiredness due to anaemia may precipitate a clinic visit but the majority of cases are detected incidentally. Hepatomegaly of 2-10cm is invariably present and examination often reveals signs of anaemia. Episodes of painless jaundice can also occur due to haemolysis, particularly in pregnant women. Laboratory investigations may confirm hypersplenism with anaemia and thrombocytopenia. High IgM levels and hepatic sinusoidal lymphocytosis will support a diagnosis of TSS but may be absent.

Diagnosis
Definite criteria for the diagnosis of TSS have been published (figure 5). However it may be difficult to
apply these rigidly in tropical situations where IgM levels and liver biopsies are not easily available and long-term follow-up of patients is unsatisfactory.

Management
The spleen usually starts to shrink after 3-6 months of continuous treatment with anti-malaria drugs such as proguanil 100mg/day. The response should be sustained and the maximum reduction will be achieved by 12-24 months. The spleen may never become impalpable and will regrow if treatment is stopped. About 20% of patients with TSS do not respond well to medical treatment even if compliance is assured. About half of these will have lymphoma and there is some evidence to suggest that TSS may transform into a malignant lymphoproliferative disorder. The role of splenectomy in TSS is not well defined because of difficulty in making a firm diagnosis and lack of follow-up data to enable mortality and post-operative infection risk to be assessed. In the 1960s, prior to established medical treatment, a few cases of TSS underwent splenectomy for debilitating symptoms and unresponsiveness to anti-malaria drugs. They showed symptomatic improvement without an increase in life expectancy. Despite the degree of splenic enlargement in TSS, rupture of the organ is rare and splenectomy
cannot be justified on the basis of size alone. Elective surgical intervention in cases of massive splenomegaly, including TSS, is only indicated in patients with severe symptoms who have not responded to drug therapy and in whom the diagnosis is in doubt.

Splenectomy in the tropics

Indications (figure 6)
These are the same as in temperate zones.

Risks
In the tropics the lack of follow-up information on patients after splenectomy precludes an accurate assessment of all the risks. In Uganda, elective splenectomy for patients with hypersplenism and spleens weighing 1.5-4.5kg had an early post-operative mortality rate of 4.8%. Long-term complications could not be recorded. The danger of splenectomy in tropical climates is twofold. Firstly, splenomegaly results in thrombocytopenia and the size of the spleen may make the procedure technically awkward; platelet and blood transfusions may be required peri-operatively and are not always readily available. Secondly, post-splenectomy patients are
particularly susceptible to bacterial infections, especially with encapsulated organisms. This produces the classical syndrome of pneumococcaemia, disseminated intravascular coagulation and shock and has a mortality of 50-80% in established cases. The risk of this syndrome following diagnostic splenectomy is not known but worldwide, in children with haemato-oncological diseases, it is 2.5%. In malarious areas there is the additional potential hazard of overwhelming malaria infection. Research with animal models suggests that preservation of a portion of spleen, preferably with arterial structures and splenic architecture intact rather than total splenectomy, may be beneficial in preventing pneumococcal infection.

Precautions

Vaccination against H. influenzae type b, N. meningitidis and S. pneumoniae is indicated prior to elective splenectomy and in those patients who have already had their spleens removed. After surgery they require prolonged anti-malarial prophylaxis and urgent empirical therapy for any febrile illness until microbiological results are available.

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Further reading


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Figure 3. Causes of massive splenomegaly

- Schistosomiasis
- Idiopathic non-tropical splenomegaly
- Tropical splenomegaly syndrome
- Chronic myeloid (and, in Africa, lymphocytic) leukaemia
- Myelosclerosis
- Kala-azar

Figure 2. Accurate bedside estimation of splenic size (optional)

Line of measurement should follow splenic contour
Figure 4. Pathogenesis of tropical splenomegaly syndrome (optional)

- Cytotoxic antibody → Suppressor T lymphocytes
- Malaria parasite → B lymphocytes
- Antigen + Mitogen → IgM
- IgM → High molecular weight immune complexes
- High molecular weight immune complexes → Splenomegaly, Hepatic sinusoids, Lymphocytosis
Figure 5. Diagnostic criteria for TSS

Major criteria

Spleen of at least 10cm
Long-term residence in a malarious area
IgM level raised to at least 2SD above local normal mean
Good response to anti-malaria drugs

Minor criteria

Liver biopsy showing hepatic sinusoidal lymphocytosis
Normal immune response to antigen challenge
Normal phytohaemagglutinin response
Hypersplenism
Lymphocyte proliferation
Familial occurrence

(Adapted from Fakunle 1981)
Figure 6. Indications for splenectomy

Definite:
- infarction
- rupture
- chronic idiopathic thrombocytopenia
- staging in Hodgkin's disease and some other lymphomas

Occasional:
- severe hypersplenism (eg. haemolytic anaemia, Felty's syndrome)
- diagnosis if cause undetermined by other methods