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Large granular lymphocyte (LGL) proliferations are being increasingly recognised.1 New classifications have been proposed to describe LGL proliferations based on immunophenotypic profiles, replacing the numerous and sometimes confusing terms that have previously been used.23 High grade lymphoproliferative disorders developing in association with LGL proliferations have been reported, and include aggressive natural killer (NK) cell leukaemia, and lymphomas of large granular lymphocytes.4-6 CD3+ LGL proliferations have been associated with low grade non-Hodgkin’s lymphoma,7 however to our knowledge, a chronic CD3− NK type LGL proliferation preceding the development of high grade B cell non-Hodgkin’s lymphoma has not been described. We report the clinical, histological, immunophenotypic, functional, and molecular characteristics of a chronic CD3− LGL proliferation that preceded the development of a high grade non-Hodgkin’s lymphoma by six years and discuss the possible causal link between the LGL lymphocytosis and B cell tumour.

Case report
A 43 year old white man was noted to have a lymphocytosis when admitted for a left inguinal hernia repair in February 1987. A full blood count showed a white cell count (WBC) of 16.4 x 10^9/L with 84% lymphocytes, the majority of which were typical LGLs, and 12% neutrophils. Haemoglobin was 15.3 g/dl and...
The patient developed symptoms of anorexia and fatigue. Further investigation showed no evidence of intra-abdominal lymphadenopathy, a normal chest radiograph, and the bone marrow aspirate and biopsy specimen showed no significant lymphocytic infiltrate. In June 1989 treatment with pulsed chlorambucil (10 mg once daily for 14 days) and prednisolone was started. Six cycles of chemotherapy were given with symptomatic improvement. The WBC fell to $4.2 \times 10^3/\text{l}$ (30% lymphocytes) but a persistent CD3− NK+ population could still be identified immunophenotypically. Low dose prednisolone (2.5 mg daily) was continued to maintain asymptomatic aminotransferase activity within the normal range but the spleen was still just palpable.

The LGL proliferation remained stable until April 1993 when the patient represented with lymphadenopathy in the left side of the neck and 7 cm splenomegaly. A full blood count showed a haemoglobin concentration of 14.2 g/dl, a platelet count of $176 \times 10^9/\text{l}$ and a WBC of $3.9 \times 10^9/\text{l}$ of which $1.2 \times 10^9/\text{l}$ were lymphocytes, many of which had LGL morphology. Repeat immunophenotyping identified a persistent CD3− NK+ population.

A lymph node biopsy was performed and was consistent with a diffuse large cell non-Hodgkin's lymphoma (see methods and results). Further imaging by computed tomography (CT) confirmed splenomegaly but no other lymphadenopathy was noted nor was there any evidence of oesophageal or gastric varices. A bone marrow trephine biopsy specimen showed focal lymphoid aggregates suggestive of bone marrow involvement by a low grade lymphoproliferative disorder. Combination chemotherapy (chlorambucil, procarbazine, vinblastine and prednisolone) was administered with rapid resolution of the adenopathy. Six monthly cycles of chemotherapy were completed by December 1993. Follow-up CT scan showed reduced but persistent splenomegaly but no nodal enlargement. The high grade non-Hodgkin's lymphoma remains in complete remission eight months after chemotherapy.

**Methods and results**

At presentation, large granular lymphocytes were easily recognised on Wright Giemsa stained smears of peripheral blood as large cells with abundant cytoplasm containing variable numbers of azurophilic granules. A bone marrow biopsy performed in 1988 showed no evidence of lymphocytosis or marrow involvement by a lymphoproliferative disorder.

Immunophenotyping of peripheral blood mononuclear cells (PBMC) obtained by Ficoll-Hypaque density gradient centrifugation was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, California, USA). Double labelling was carried out using a panel of monoclonal antibodies directly conjugated to fluorescein or phycoerythrin (Becton Dickinson). At presentation in 1987, 85% of peripheral blood lymphocytes had the following phenotype: CD3−, CD4−, CD8−, platelets $270 \times 10^9/\text{l}$. There was no history of infections, weight loss or night sweats, and no other relevant past medical history. Physical examination was unremarkable.

Over the next 12 months, the LGL lymphocytosis persisted between 10.8 and $13.7 \times 10^9/\text{l}$. The patient was rheumatoid factor positive, but other autoantibodies were negative and serum immunoglobulin levels were normal. The immunophenotypic profile of the LGLs was as follows: CD2+, CD3−, CD4−, CD8−, CD16+, CD56+, CD57+, CD11b+ (see methods and results). Splenomegaly and deranged liver function with a raised aspartate aminotransferase of 107 U/l (normal range $<37 \text{U/l}$) was noted. Antibodies to hepatitis A, B and C, HIV I and II, cytomegalovirus, Epstein–Barr virus, and human T cell lymphotropic virus I were not detected. A liver biopsy was performed which revealed a non-specific active chronic hepatitis, with no evidence of a malignant lymphoid or leukaemic infiltrate.
Natural killer activity (expressed as per cent lysis of 2000 targets) of mononuclear cells from the patient and from a normal control with known high NK activity. Absolute counts of CD3-, CD16+, CD56+ per E:T ratio are shown in brackets (× 10^4/1).

<table>
<thead>
<tr>
<th>Subject</th>
<th>E:T ratio</th>
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<tr>
<td>Patient</td>
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The normal range of specific NK activity has been determined to be 15–35% in this system at an E:T ratio of 100:1.

CD57+. When repeated on several occasions in 1988 and 1989, the LGL phenotype was consistently recognised as CD2+, CD3−, CD4−, CD8−, CD16+, CD56+, CD57+, CD11b+. Following treatment with chlorambucil, the absolute lymphocytosis decreased although a population of LGLs was still recognisable morphologically, and approximately 40% of the peripheral blood lymphocytes had the phenotype of CD3−, NK+ cells described earlier. This persisted up to April 1993 and after treatment for the high grade non-Hodgkin’s lymphoma a smaller CD3−, NK+ population (30%) could be identified. Absolute numbers of CD4+ T cells were low and ranged from 0–16 × 10^4/l from 1988 to 1993; only on one occasion in August 1988 were normal CD4+ numbers recorded.

The neck lymph node biopsy was received fresh and a portion was snap frozen in liquid nitrogen. Fixed, embedded sections were stained with haematoxylin and eosin and with a panel of monoclonal antibodies directed against CD20, CD45, CD3, CD45RO, k and λ light chains (Dako), CD45RA, MB2, CD43 (Eurodiagnostics), and CDw75 and CD74 (Novacastra). Frozen sections (8 μm) were stained with the following monoclonal antibodies: CD2, CD3, CD4, CD8, CD11b, CD11c, CD16, CD19, CD22, CD23, CD56, and CD57 (Becton Dickinson; diluted 1 in 5).

A standard three step avidin-biotin complex (ABC) technique utilising the Streptavidin ABC complex/horseradish peroxidase duet kit (Dako) was used for all the immunohistochemical staining with diaminobenzidine as the chromagen and Meyer’s haematoxylin as the counterstain. The lymph node architecture was totally effaced by a diffuse infiltrate of large lymphoid cells with open vesicular nuclei, consistent with centroblasts. The tumour cells showed strong, positive epimembranous staining with CD45, CD20, MB2, CD74, and CD22, consistent with a high grade centroblastic B cell non-Hodgkin’s lymphoma. No light chain restriction could be demonstrated.

A staging marrow trephine biopsy performed in April 1993 revealed a number of lymphoid aggregates suggestive of marrow involvement by a low grade lymphoproliferative disorder but LGLs were not seen in the marrow smears or trephine biopsy sections. Chromosomal analysis by standard G-banding techniques of bone marrow aspirate derived metaphases showed a normal 46 XY karyotype.

The NK functional activity of the patient’s PBMC was assessed in April 1993. Specific lysis of K562 was used as a measure of NK cytotoxicity. A flow cytometric assay8 was used and a normal range of NK activity established on samples from 60 normal donors. The functional NK cytotoxicity of the patient’s PBMC was greatly elevated (table). The activity of the patient’s PBMC was more than twice as high as the control at higher effector : target (E : T) ratios and four times as high at lower E : T ratios.

Southern blot analysis for immunoglobulin and T cell receptor (TCR) gene rearrangements was performed on DNA extracted from the lymph node biopsy and PBMC obtained at the same time. DNA was extracted from the lymph node and PBMC and digested with restriction enzymes. Southern transfer was performed using Amersham Hybond C membranes. The hybridisation probes used were pH60 from the J region of the TCRγ locus,^8 B10BB1 from the C region of the TCRβ locus,^10 c3.2 from the constant region of the immunoglobulin κ light chain locus,^11 R17CK from the constant region of the immunoglobulin κ light chain locus region,^12 and an immunoglobulin heavy chain gene probe comprising a 0.95 kilobase BstElI fragment derived from the 3′ end of the JH region. The lymph node and PBMC sample obtained in April 1993 were in germline configuration for the TCRβ- and γ chain gene loci. Both samples showed rearrangements of the immunoglobulin heavy chain gene locus, but with distinct rearrangements being seen in the two samples, indicative of separate and distinct B cell populations (figure). No rearrangements of immunoglobulin κ or λ chain genes were detected. A third sample, comprising PBMC obtained in March 1994, was also examined. This sample showed the presence of a small clonal population of B cells possessing rearrangements of the immunoglobulin heavy chain distinct from the populations seen previously in the peripheral blood and node.

**Discussion**

LGL proliferations encompass a spectrum of disorders that include transient reactive, persistent reactive, premalignant, or truly clonal disorders that have a varied clinical course.13,14 Immunophenotypically, LGL proliferations can be divided into two major types: CD3− and CD3+. CD3+ LGLs are T cells that have rearranged their TCR genes and are thought to represent activated cytotoxic T cells responsible for specific major histocompatibility complex restricted cytotoxicity.14 CD3− LGL proliferations have a NK+ phenotype recognised by the expression of certain surface antigens, and activity in vitro assays of NK cell function.

The clinical course and features of LGL proliferations are very heterogeneous.1 Certain well characterised entities are now recognised—for example, CD3+ T LGL with LG lymphocytosis, neutropenia and recurrent infections seen in association with rheumatoid disease or Felty’s syndrome.14 CD3+ LGL proliferations may also be associated with chronic infections, viral hepatitis, tuberculosis, epitheliod carcinomas, and non-Hodgkin’s lymphoma.17
Proliferation of CD3—LGLs preceding development of non-Hodgkin’s lymphoma

CD3—LGL proliferations, however, are much less common.

In persistent CD3+ LGL proliferations chronic reactive polyclonal states can be differentiated from malignant clonal disorders by identifying clonal rearrangements of TCR-β- or -γ chain gene loci. In CD3— LGL proliferations karyotypic abnormalities have been sought as markers of clonality. An alternative approach in women is to study X linked polymorphisms using probes for phosphoglycerate kinase or M27β. Only 11 patients with CD3— LGL proliferations were identified in a recent review that fulfilled predefined criteria for clonal proliferations, and the clinical presentation of CD3— NK+ LGL leukaemia was quite different from CD3+ T LGL leukaemia.

In this case the CD3— LGL proliferation showed no evidence of rearranged TCR-β or -γ chain genes. Bone marrow karyotypic analysis failed to show a chromosomal abnormality. No T cell or chromosomal marker of clonality was therefore identified. It is therefore uncertain whether the lymphocytosis was a chronic reactive proliferation or a truly malignant one. The reactive polyclonal nature of some longstanding CD3— NK+ LGL proliferations has been demonstrated using X linked polymorphism techniques. However, certain features in this case support the contention that the LGL proliferation may have represented a low grade malignancy. The long-standing splenomegaly was not thought to be due to liver disease as the liver biopsy specimen showed no evidence of cirrhosis and the CT scan failed to demonstrate evidence of portal hypertension. The splenomegaly may therefore have been a manifestation of LGL infiltration. Splenomegaly was not reported in the six cases of chronic reactive CD3— NK+ LGL reported by Nash et al. The bone marrow biopsy specimen taken in April 1993 also showed changes consistent with involvement by a low grade lymphoproliferative disorder.

While non-Hodgkin’s lymphoma and lymphomas of LGLs have been described in CD3+ LGL proliferations, the occurrence of high grade B cell non-Hodgkin’s lymphoma following a chronic CD3— NK+ LGL proliferation has not, to our knowledge, been reported previously.

Is the relation between the chronic LGL proliferation and the subsequent development of the high grade B cell lymphoma causal or purely fortuitous? It seems unlikely that the non-Hodgkin’s lymphoma was related to the short period of exposure to low dose alkylating agents in 1989. Tumour surveillance is one function of NK cells; however, the non-Hodgkin’s lymphoma developed despite high in vitro activity being demonstrated in functional NK cell assays. There was however evidence of T cell abnormalities with low levels of CD4+ lymphocytes being noted during most of the time the patient was under review. An association between the clonal B cell population detected in the peripheral blood and the tumour is not proven in this case. It is, however, of interest to note that B cell dysfunction manifested as multiple autoimmune body, polyclonal hypergammaglobulinaemia and concurrent development of low grade B cell lymphoproliferative disorders has been described in CD3+ LGL proliferations. Bassan et al postulated that normal B cell regulation mediated by T and NK cells is lost in some LGL proliferations which may lead to the eventual development of clonal B cell disorders. The identification in this case of transient clonal B cell populations in the peripheral blood would support this suggestion. Patients with chronic persistent LGL proliferations both of CD3— and CD3+ phenotypes may be at risk of developing manifestations of B cell dysregulation which may include B cell lymphoproliferative disorders.