MECHANISMS OF ACTION OF INTERFERON-ALPHA IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

BY

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ABSTRACT

Interferon-alpha is able to produce partial responses in patients with early stage B cell chronic lymphocytic leukaemia (CLL), but the responses are highly variable, both in extent and duration. This thesis explores the possible cellular bases for these clinical responses and their heterogeneity, and addresses the adhesive properties of CLL cells, in relation to their homing behaviour, as well as the cytokine regulation of CLL cell survival and apoptosis, and the role of effector cells, using both in vitro and in vivo studies. In a current clinical trial conducted in patients with early stage CLL, interferon-alpha produced partial responses, in terms of a significant reduction in lymphocyte counts, and the development of anti-interferon antibodies correlated with loss of clinical response. There was little evidence for T cell activation in patients receiving interferon, but there were significant rises in serum levels of macrophage colony-stimulating factor (M-CSF) and neopterin, suggesting that mononuclear cell activation may be involved in the generation of haematological responses to interferon-alpha.

Normal lymphocytes undergo well regulated patterns of recirculation in vivo, and abnormal patterns may be involved in the pathophysiology of CLL. CLL cells have abnormal expression of adhesion and homing molecules, and interferon-alpha was able to induce surface expression of the lymphocyte homing receptor, L-selectin. The binding properties of CLL cells to cultured human high endothelium, and umbilical vein-derived endothelium, in the context of cytokine stimulation, were examined.
CLL cells express the bcl-2 oncoprotein, but do not possess the t(14;18) translocation. *In vitro*, CLL cells demonstrate high levels of spontaneous apoptosis, and interferon-alpha was able to increase expression of the bcl-2 protein, both *in vitro* and *in vivo*, and to protect these cells against both spontaneous and induced apoptosis.

Several cytokines have been implicated in the autocrine control of growth and differentiation of CLL cells. In this study, cytokines such as IL-6 and TNF, as well as soluble CD23, were detected in the peripheral blood of patients with CLL, however, the question of their potential role as autocrine growth factors remains unresolved. The ability of several cytokines, including interferons-alpha and gamma, and the interleukins to protect CLL cells against apoptosis may be relevant to this question.
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1.1 Chronic Lymphocytic Leukaemia

*History and Definitions*

During the middle of the nineteenth century, the combined efforts of many workers in several different countries using clinical and microscopic evidence lead to the recognition of leukaemia as a disease. The term leukaemia was introduced by Virchow, and it was he who first distinguished between 'splenic' leukaemia and 'lymphatic' leukaemia, terms which correspond to modern definitions of chronic myeloid leukaemia and chronic lymphocytic leukaemia (CLL) respectively (Virchow 1856). However, it was not until 1903 that Turk established diagnostic criteria, and identified features that distinguished it from the lymphomas (Turk 1903). By 1924, the first detailed clinical description of CLL was available (Minot & Isaacs 1924). Despite extensive work on the nature and treatment of the disease, major advances in the understanding of CLL were hampered by ignorance about the function and physiology of normal lymphocytes. It was not until the early 1960's that Gowans was able to establish the central importance of the lymphocyte in both humoral and cell-mediated immunity (Gowans & McGregor 1965). Using adoptive transfer experiments, Gowans was able to demonstrate that the small lymphocyte could become an antibody synthesizing cell and an effector cell in transplantation reactions. It soon became clear that the small lymphocytes in CLL were functionally abnormal. These revelations lead to the suggestion that CLL was characterised by the progressive accumulation of small
lymphocytes with a mature morphological appearance, but which were functionally incompetent (Galton 1966, Dameshek 1967). This definition is still valid in the broadest sense today. However, the explosion of information about the immunology, biochemistry and physiology of normal lymphocytes means that our understanding of these abnormal lymphocytes has advanced considerably, and is still expanding. Increased understanding of the aetiology, pathogenesis and immunology of CLL should enable rational therapeutic strategies to be established in the future.

**Aetiology and pathogenesis**

It is now widely accepted that the pathogenesis of human cancers is a multi-step process that depends upon the interaction of several factors. These may include genetic susceptibility, genetic mutations, immunodeficiency, viral infection, exposure to radiation, and other environmental factors. For example, the t(14;18) chromosome translocation occurs in approximately 85% of follicular lymphomas (Fukuhara et al. 1979, Yunis et al. 1987), and the t(9;22) translocation occurs in 90% of chronic myeloid leukaemias (Kurzrock et al. 1988). These appear, however, to be neither necessary nor sufficient to cause the disease. Clearly in both cases, some patients with clinical disease lack the translocation, even though their clinical manifestations may be indistinguishable from patients whose cells have the translocation. Furthermore, apparently identical translocations may be found in diseases with completely different clinical presentations. For example, 10% of adults with acute lymphoblastic leukaemia have an identical t(9;22) translocation to those with chronic myeloid leukaemia (Kurzrock et al. 1988).
Transgenic mice expressing the bcl-2-immunoglobulin mini-gene that mimics the t(14;18) translocation in follicular lymphomas demonstrate an accumulation of polyclonal, small resting B-cells (McDonnell et al 1989, McDonnell et al 1990), but do not develop monoclonal malignant lymphomas for some time, suggesting the necessity for a second event to precipitate malignant change. These points must be borne in mind when considering factors which are associated with CLL. Furthermore, it is often difficult to determine whether particular changes occur as a cause or consequence of the disease.

Race
CLL is the commonest leukaemia in the Western world, accounting for approximately 30% of all leukaemias and 70% of all lymphoproliferative disorders. However, CLL is extremely rare in Asians (Finch & Linet 1992). The differences between populations in the incidence of CLL show greater variation in CLL than other leukaemias. The low incidence of CLL in the East is undoubtedly related to underdiagnosis, either due to lack of physician access, poor facilities for diagnosis, low level of diagnosis of indolent disease or misdiagnosis. It has also been suggested that the low incidence of CLL in the third world may reflect the shortened life span in these countries (Fleming 1990). However the incidence of CLL is also low in Japan, where life expectancy is similar to that in Western countries (Catovsky & Foa 1990). Furthermore, immigrant populations in the USA parallel international patterns, with Asians (Chinese, Japanese, Phillipinos) showing a very low incidence (Finch & Linet 1992). These findings suggest a genetic susceptibility in white Caucasian populations.
Age
It has been apparent for some time that there is a strong relationship of aging to the development of CLL. Over 90% of patients are over 50 years old and more than two thirds are over 60 (Catovsky et al 1989). CLL is rare in patients under 40. It is not clear whether this is due to changes in the immune system with age that may predispose to neoplastic transformation (Gross et al 1965), or whether it reflects pathogenesis more directly.

Sex
Incidence of CLL is consistently higher in males than females. The male: female ratio varies from country to country, with a ratio of 4.7 in Australia in contrast to one of 1.0 in Columbia (Finch & Linet 1992)

Irradiation
Exposure to radiation has been recognized as a factor in the aetiology of several types of leukaemia, but all studies so far suggest that radiation does not increase the risk of CLL (Mole 1990).

Environmental factors
Exposure to a variety of chemicals and other environmental agents has been implicated in the aetiology of many leukaemias, especially some acute leukaemias. Reports have demonstrated a small increase in incidence of CLL on exposure to benzene and other solvents, but the evidence is weak (Linet & Blattner 1988). Similarly, slightly elevated risks have been shown for some farm workers, those with exposure to wood and wood products, and
male hairdressers (Linet & Blattner 1988). However, most studies have only examined small numbers of cases of CLL and it is difficult to exclude other confounding factors.

Familial incidence

The results of a number of studies have demonstrated that the risk of relatives of patients with leukaemia also developing the same leukaemia is highest in patients with CLL (Gunz 1977). In two large studies in Australia and New Zealand, the incidence of CLL in first-degree relatives was three times that of the general population (Gunz & Veale 1969, Gunz et al 1975), with no significant increase in the incidence of other malignancies. There was no clear cut pattern of inheritance and consanguinity did not appear to be a factor. This might suggest that environmental factors are important. However because CLL occurs typically in patients over 50 years of age, patients with familial leukaemia would not have shared common environmental factors for decades. It is possible that there might be a prolonged period of preleukaemia in some of these patients (Gale 1991). However, in healthy relatives of patients with CLL there were no abnormalities in blood counts or lymphocyte morphology (Gunz & Veale 1969). More recently it has been suggested that close relatives of patients with CLL may have an increased incidence of auto-immune disease (Conley et al 1980).

Cytogenetics and oncogenes

Studies of chromosomal abnormalities in CLL were beset by technical difficulties in obtaining suitable metaphases for examination, and the uncertainty as to whether the metaphases
arose from the malignant clone. However, new polyclonal B cell activators produce suitable metaphases. In about 50% of patients with CLL, chromosomal abnormalities can be detected. The commonest abnormalities are trisomy 12, and deletions of the long arms of chromosome 11 or 13 with translocation to the long arm of chromosome 14 (Juliusson & Gahrton 1990). However the reciprocal observation that in half of the CLL patients no chromosomal abnormality can be detected, suggests that a specific CLL gene is unlikely. Furthermore, these chromosome abnormalities are not specific for CLL. Interestingly the structural abnormalities on chromosome 13 may involve the retinoblastoma gene (Juliusson et al 1991). However, in one study no deletion of the retinoblastoma gene was found (Raghoebier et al 1991). Oncogenes such as bcl-1, bcl-2 and c-myc have been found to be rearranged in some patients with CLL (Raghoebier et al 1991, Rechavi et al 1989), but no consistent oncogene associations have been demonstrated.

**Viruses**

While there is no direct evidence that CLL is of viral origin the prospect remains attractive. The long prodromal period between the presumed leukaemogenic insult and the clinical manifestation of the disease reflects the course of many virally associated malignancies. For example the long period between infection with HTLV-1 (human T-cell leukemia virus-1) and the development of acute T-cell leukaemia (Blattner 1990), or the latency period between infection with Epstein-Barr virus (EBV) and the development of Burkitts Lymphoma bear some resemblance to the long, indolent course of CLL. However, despite similarities with
diseases caused by HTLV-1, EBV and many other viruses there is no clear viral cause for CLL.

Autoimmunity

Although hypogammaglobulinaemia is often seen in CLL (Ben-Bassat et al 1979), CLL has also been associated with a variety of auto-immune disorders including pernicious anaemia, rheumatoid arthritis, myasthenia gravis and thyroid disorders (Conley et al 1980). Auto-immune haemolytic anaemia (AIHA) and idiopathic thrombocytopenic purpura (ITP) are both well known complications of CLL (Catovsky & Foa 1990). These auto-antibodies are normally IgG and are therefore presumably not the product of the malignant clone (Catovsky & Foa 1990). However, it has been reported that CLL cells do secrete autoantibodies (Broker et al 1988, Sthoeger et al 1989), and in some patients with CLL, monoclonal IgM expressing the same light chain as the CLL cells can be demonstrated in the serum (Deegan et al 1984). These antibodies are produced by a restricted group of variable region genes that have not undergone somatic hypermutation (Kipps et al 1991). These antibodies express cross reactive idiotypes (CRI) that are commonly present on autoreactive IgM molecules (Kipps et al 1991). It is conceivable that the expression of these autoantibodies could lead to chronic stimulation of the cell and increase the likelihood of malignant transformation. Alternatively the association between CLL and autoimmune phenomena may reflect underlying abnormalities in T-cell immunology common to both types of disease.
Immunology

The CLL cell

Many studies have now used monoclonal antibodies for detailed analysis of the cell surface phenotype of the CLL cells. In 95% of cases, CLL develops from the malignant transformation and clonal expansion of a B lymphocyte, while in less than 5% of cases, the malignant clone is of T cell lineage. The work in this thesis is concerned only with B lineage CLL (CLL). Phenotype studies on CLL cells have demonstrated the heterogeneous nature of the disease, and have also been used to assign a putative normal cellular counterpart to the CLL cell. Most CLL cells express low levels of surface IgM, with or without IgD, the pan B cell antigens CD19, CD20, CD21, and CD24, as well as the T-cell associated antigen CD5 (Mulligan 1990). Table 1.1 summarizes the surface phenotype of CLL cells. This phenotype resembles that found on foetal B cells from spleen and blood, and on a minor B cell population in normal adult lymph node and blood. These observations have lead to the suggestion that these B cells are the normal cellular counterpart of the CLL cell (Mulligan, 1990).

Table 1.1. Surface features of CLL cells

<table>
<thead>
<tr>
<th>Heavy chain, usually μ, or μ and δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light chain, either κ or λ</td>
</tr>
<tr>
<td>Complement receptors (C3d/EBV-CD21)</td>
</tr>
<tr>
<td>Fc receptor for IgG</td>
</tr>
<tr>
<td>la antigens (HLA-DR, DC, variable DQ)</td>
</tr>
<tr>
<td>B-cell associated antigens (CD19, CD20, CD21, CD24)</td>
</tr>
<tr>
<td>Mouse erythrocyte receptors</td>
</tr>
<tr>
<td>T-cell associated antigen (CD5)</td>
</tr>
</tbody>
</table>

23
On the other hand, CLL cells also express the activation antigens CD23 and CD25 (Freedman and Nadler 1990). The presence of activated B cells and increased numbers of CD5 positive B cells in the peripheral blood of patients with autoimmune disorders (Hayawara & Hardy 1988) suggest that CLL cells may correspond to a minor subpopulation of activated B cells. This suggestion is supported by the observation that phorbol myristate acetate (PMA) can induce CD5 expression on a proportion of normal B cells (Freeman & Nadler 1990). However, studies on immunoglobulin gene rearrangements in CLL cells demonstrate that the immunoglobulin genes in CLL cells have not undergone somatic hypermutation (Kipps et al 1991). This suggests that the B cells have not undergone affinity maturation in response to antigen. Furthermore, the antibodies produced from these rearrangements are frequently autoreactive, and often express cross reactive idiotypes (CRI) present on other autoantibodies (Kipps et al 1991, Dighiero & Borche 1991). Chronic stimulation of autoreactive cells may therefore allow secondary neoplastic events to occur and precipitate the development of CLL. Alternatively, the disease may arise from ineffective attempts at clonal anergy of the autoreactive cells.

Functionally, CLL cells appear to demonstrate a variety of abnormalities. However, these studies are often difficult to interpret because of the problems of isolating pure populations of malignant cells, and contamination with residual normal B cells, T cells or monocytes can have profound effects on interpretation of differentiation and proliferation experiments. The low levels of surface immunoglobulin, absence of capping and the inability to stimulate mixed lymphocyte cultures appear to be linked to
defects in cell membrane motility (Foon & Gale 1988). Responses to B cell mitogens such as pokeweed mitogen, lipopolysaccharide and Epstein-Barr virus are abnormal, as are responses to stimulation with antibodies directed against surface immunoglobulin, CD20 or CD40 (Freedman 1990). Stimulation with PMA can induce CLL cells to acquire features suggestive of differentiation, such as plasmacytoid morphology, increased IgM secretion and in some cases the switch to IgG secretion (Freedman 1990). Cytochalasin B is reported to be a potent mitogen for CLL cells.

Responses to cytokines have been extensively studied but much of the data remains contradictory. Thus while interleukin 2, tumour necrosis factor and interferon-alpha have been reported to induce or support differentiation and proliferation in CLL cells, it seems likely that their responses are impaired when compared to normal B cells (Foon & Gale 1988, Freedman 1990).

**T cells in CLL**

Although the role of immunoregulatory mechanisms in the pathogenesis of CLL remains obscure, the involvement of T-cell abnormalities in the disease remains likely. The absolute numbers of T-cells are increased in CLL, while the numbers are decreased in comparison to the numbers of B-cells. Both CD4 positive and CD8 positive T-cells are increased, but the increase in CD8 positive cells is several times that of the CD4 positive cells, leading to a reduced CD4/CD8 ratio (Zaknoen & Kay 1990). These effects appear to be more pronounced in patients with advanced disease. Similarly, T-cell function appears to deteriorate with disease progression in CLL, and defects may only be detected in
patients with advanced disease. This suggests that abnormal T-cell function in CLL is a secondary event associated with disease progression, and is not a primary event in the leukaemic process. However, the T-cell defects almost certainly play a role in the immuneparesis in advanced disease, and thus may influence the clinical course.

A number of T-cell functions have been studied in CLL (reviewed in Foon & Gale 1988, Freedman 1990, Catovsky & Foa 1990). Responses to mitogens may be normal or decreased, mixed lymphocyte reactions are usually impaired and T-cell colony growth is reduced. In pokeweed mitogen driven systems, CD4 helper cell activity may be decreased, and conversely, CD8 suppressor activity is increased in patients with advanced disease. However, in patients with early disease both helper and suppressor activity may be normal (Freedman 1990). Production of IL-2 and interferon-gamma appears to be normal in CLL (Catovsky & Foa 1990). While levels of the soluble IL-2 receptor are raised in CLL (Semenzato et al 1987), it seems likely that this is a product of the malignant B cells.

Numbers of natural killer (NK) cells are increased in CLL, but both NK cell activity and lymphokine activated killer (LAK) cell activity are impaired (Foa et al 1990, Jewell et al 1992). These results are particularly interesting in view of the extensive data that support a regulatory role of natural killer cells on B cells and immunoglobulin production (Abruzzo & Rowley 1983, James & Ritchie 1984, Commes et al 1990).
Treatment

In many patients with CLL, the disease runs a benign course, and prolonged survival is possible, with minimal or no symptoms. Staging systems have been useful in identifying the small group of patients whose disease is more advanced, and who have a median survival of only 30 months. One such system is the Binet system (Table 1.2, Binet et al 1981).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical Features</th>
<th>Survival (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>haemoglobin &gt;10g/dL, platelets &gt;100X10^9/L; and &lt; 3 areas involved*</td>
<td>&gt;120</td>
</tr>
<tr>
<td>B</td>
<td>haemoglobin &gt;10g/L, platelets &gt;100X10^9/L; and &gt; 3 areas involved</td>
<td>61</td>
</tr>
<tr>
<td>C</td>
<td>haemoglobin &lt;10g/dL or platelets &lt;100X10^9/L, or both (independently of the areas involved)</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 1.2. Binet staging system for CLL (Binet et al 1981)

* Five areas include the cervical, axillary and inguinal lymph nodes, the spleen and the liver

Optimal treatment strategies for patients with CLL have yet to be devised, and permanent remissions are rare. Most treatment strategies have been reserved for the group of patients with advanced, or progressive disease, as evidenced by rapidly enlarging or painful lymphoid masses, autoimmune cytopaenias, and progressive bone marrow failure due to disease involvement. Given the potential for complications with all forms of therapy, and the lack of substantial evidence for significant survival benefit in patients treated during the early stages of their
disease, treatment of patients with early stable disease is not normally undertaken. On the other hand, the clinical course of patients with early or intermediate stage disease is unpredictable, in that a proportion will progress rapidly, and therefore, would presumably benefit from treatment early on in their disease. Unfortunately, staging systems do not distinguish those patients with early stage disease who will progress rapidly, from those who will continue to run an indolent course. The rationale for treating CLL in early stage would therefore be in an attempt to benefit those patients who are likely to have a more aggressive disease. In addition, most treatment modalities appear to produce better responses in early disease. The search for effective therapy of early stage disease, however, presents a number of problems. Firstly, assessment of efficacy is difficult. Because the median survival of patients with early stage disease is greater than 10 years, use of survival as an indicator of advantage for treatment is impractical. Most response criteria therefore rely on the lymphocyte count to assess the effectiveness of treatment. Secondly, it is difficult to decide on the most appropriate schedule for therapy; whether to use continuous or pulsed therapy; the role of maintenance therapy and the choice of agent; the use of single agents versus combinations of treatment modalities. These issues remain to be resolved.
Chemotherapy

A variety of chemotherapeutic agents have been used in CLL (Han & Rai 1990, Cheson 1992). Single agent chemotherapy has relied upon the alkylating agents, chlorambucil and cyclophosphamide. Responses to these agents vary. Up to 70% of patients with early stage CLL respond to chlorambucil, although responses may be as low as 30% in advanced disease. Responses to cyclophosphamide are similar, but most experience has been in combination therapy, normally with corticosteroids such as prednisolone and one or more other cytotoxic agents such as vincristine (COP) or vincristine plus adriamycin (CHOP). Although such combination regimens may produce a higher response rate, particularly in advanced disease, it is controversial whether overall survival rates are any better when compared with chlorambucil alone, and it is clear that none are capable of producing cure in CLL. Furthermore, the clinical benefit of treatment in in early stage disease remains unclear. Studies have failed to demonstrate that patients with early stage disease who were treated with chlorambucil had a survival advantage over patients who received no treatment (French Cooperative Group on CLL 1990).

Several new agents are now available for the treatment of CLL. The purine analogues, 2-chlorodeoxyadenosine, 2-deoxycoformycin and fludarabine have all been shown to be effective in heavily pretreated patients with advanced and resistant disease (Dillman et al 1989, Piro et al 1988). Most clinical experience has been with fludarabine and response rates of up to 75% in previously untreated patients have been noted (Keating et al 1991).
Radiation
All lymphocytes are highly susceptible to the effects of radiation and CLL cells are no exception. However, despite several reports of efficacy, splenic irradiation has not been adopted as first line therapy (Catovsky & Foa 1990).

Bone marrow transplantation
Perhaps not surprisingly, only a few cases of allogeneic bone marrow transplantation for CLL have been reported (Michallet et al 1991). Although the results were promising, the treatment related morbidity and mortality make this unlikely therapy for the majority of patients. The availability of new drugs which are able to induce complete remissions in CLL raise the possibility of autologous transplantation following chemotherapy.

Biological therapy
CLL appears to be an ideal candidate for immunotherapy. It is a slowly progressing disease with recognised immunological defects, and has response criteria which are easily assessed. However, the results have been disappointing. Therapy with a variety of monoclonal antibodies and their derivatives have only produced rare, transient responses (Cheson 1992). Antibodies to CD5 or CD19 have been used alone or conjugated to ricin, and anti-idiotypic antibodies alone or conjugated to radio-isotopes have also been employed with disappointing results. Similarly studies using IL-2 have also failed to produce convincing responses. The greatest experience with biological therapy in haematological malignancies has been with interferon-alpha. Successful treatment of hairy cell leukaemia with interferon-
alpha (Quesada et al 1984) stimulated an interest in the potential use of interferon-alpha in CLL. Early studies in advanced disease were disappointing (Foon et al 1985), but several studies have now shown that interferon-alpha can be an effective therapy in patients with early stage CLL (O'Connell et al 1986, Pangalis et al 1988, Rozman et al 1988, Ziegler-Heitbrock et al 1989).

1.2 Interferon-alpha

Interferons were first described as agents able to confer resistance to viral infections, but are now known to have a variety of biological activities, including anti-tumour effects (reviewed in De Maeyer & De Maeyer-Guignard 1988). Three classes of interferon have been defined. Interferon-alpha and interferon-beta share the same receptor, but there is only about 30% homology between the proteins. Interferon-gamma shares only 10% homology with interferon-alpha, is only produced by T-cells and binds to a distinct receptor. The interferons share many biological functions, but their anti-tumour effects can be broadly grouped into two categories; direct effects on growth and differentiation of the malignant cells; and effects due to modulation of the immune response. These categories are not mutually exclusive, however. Direct effects on the malignant cell may alter their vulnerability to immune mechanisms, eg modulation of MHC and adhesion molecule expression.

Direct effects of interferon-alpha on malignant cells

Following the description of interferons as anti-viral agents, there was much interest in the effects of interferons on animal
tumours of known viral aetiology. Inhibition of tumour growth was initially seen in a variety of virally induced tumours, but it soon became clear that interferon could also inhibit growth of tumours induced chemically, and those that arose spontaneously (Gresser & Tovey 1978). It was also demonstrated that interferons could inhibit the growth of many tumour cells in vitro, and could also affect differentiation of these cells. Much interest was focused on the cellular basis for these observations, and their relationship to those events responsible for the development of the antiviral state. However, the great diversity of the effects of interferon on cellular events has made a comprehensive understanding of the direct effects of interferon on malignant cells extremely challenging. Firstly, not only do malignant cells differ from normal cells in the pattern of activation of the cellular events in response to interferon-alpha, but they also vary in their susceptibility to the effects of these events. Secondly, these differences may not only be evident between different types of malignant cells, but also within apparently clonal populations. Finally, it is still not clear which effects are a result of the primary changes induced by interferon, and which occur due to secondary responses resulting from intermediate proteins produced both within the cell and in a paracrine fashion from other cells. It is clear that interferons act as multifunctional gene activators and can induce synthesis of approximately 24 proteins (De Maeyer & De Maeyer-Guignard 1988). Some of these are known to be associated with the antiviral state, such as (2'-5')-oligoadenylate synthetase and the interferon-induced protein kinase. Although these proteins are induced by interferon in malignant cells, they are also induced in
malignant cells resistant to the growth inhibitory effects of interferon (Taylor-Papadimitriou & Rozengurt 1985). These results suggest that the antiviral state and growth inhibition effects operate via different mechanisms.

Many of the other proteins induced by interferons await characterisation, but no convincing evidence is available to implicate them in the control of malignant cell proliferation and differentiation. The induction of proteins of the major histocompatibility complex has been demonstrated, and while this does not directly lead to growth inhibition, it may lead to increased susceptibility of the tumour cells to effector functions of the immune system (see below). Interferons may also exert effects by regulating the expression of certain oncogenes (Jonak & Knight 1987, De Maeyer & De Maeyer-Guignard 1988, and see Chapter 8), but our understanding of the processes involved in these effects is hampered by our poor understanding of the physiological role of the corresponding proto-oncogenes in normal cells. However, there is now evidence that interferon may act directly on proteins that control the cell cycle by altering phosphorylation of these products (Thomas 1989).

**Immunomodulatory effects of interferons in malignancy**

The early studies on the effects of interferon on tumour cell growth had demonstrated that interferon could inhibit cell proliferation both *in vitro* and *in vivo*. However, it soon became apparent that some malignant cells that were resistant to the effects of interferon *in vitro*, were inhibited by interferon *in vivo* (Taylor-Papadimitriou & Rozengurt 1985). These observations implicated other mechanisms in the anti-tumour effects of
interferon, and it is now known that interferons have a number of effects on the immune system. Interferon-alpha increases the expression of MHC class I antigens on a variety of malignant cells, and this may reduce their oncogenic potential by increasing their susceptibility to cytotoxic T cells. Interferon-alpha may also induce the expression of MHC class II antigens on some tumour cell types, eg hairy cell leukaemia (De Maeyer & De Maeyer-Guignard 1988).

In addition to rendering the tumour cells more susceptible to immune effector mechanisms, interferon-alpha also enhances effector cell function. Interferon-alpha enhances monocyte/macrophage phagocytic and cytotoxic activity, and also increases the expression of Fc receptors for IgG on the surface of both monocyte/macrophages and neutrophils (Balkwill 1985). Interferon-alpha also increases the cytolytic activity of T-cell lines in vitro, and has dose-dependent stimulatory and inhibitory effects on B-cell and antibody production (De Maeyer & De Maeyer-Guignard 1988). Natural killer (NK) cells are particularly sensitive to activation by interferon-alpha, and interferon-alpha also generates lymphokine activated killer (LAK) cell activity (Trinchieri 1989, Jewell et al 1992). However, interferon-alpha also induces resistance to NK activity in many cells types (De Maeyer & De Maeyer-Guignard 1988). It was suggested that normal cells were more readily protected by interferon than malignant cells but this no longer appears tenable. It was hoped therefore that therapy with interferon-alpha would not only have anti-neoplastic effects, but may also have the potential to correct the immuneparesis found in many malignancies.
Interferon-alpha and human malignancy

Following encouraging results in transplantable tumours in animals (Gresser 1972), and having at least potential explanations for the mode of action in malignant disease, there was much enthusiasm for the use of interferon-alpha in the treatment of human cancer. However, it was not until the advent of recombinant DNA technology, that sufficient supplies of interferon were available for proper clinical trials. Interferon-alpha has now been used in a variety of human malignancies (Kirkwood & Ernstoff 1984, Krown 1986, Goldstein & Laszlo 1986), and although early results were encouraging, interferon-alpha has yet to become licensed for first-line therapy in more than a handful of malignant diseases (see Chapter 3).
CHAPTER 2
MATERIALS AND METHODS

2.1 GENERAL MATERIALS

**PLASTICS**
All plastics used were of tissue culture grade polystyrene unless stated otherwise.

<table>
<thead>
<tr>
<th>Sterile disposable plastics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mL universal containers</td>
<td>Sterilin Ltd.,</td>
</tr>
<tr>
<td>250 mL specimen containers</td>
<td>Feltham, UK.</td>
</tr>
<tr>
<td>7 mL bijou bottles</td>
<td></td>
</tr>
<tr>
<td>15 cm² petri dishes</td>
<td></td>
</tr>
<tr>
<td>50 mL polypropylene tubes</td>
<td></td>
</tr>
<tr>
<td>25 cm² tissue culture flasks</td>
<td>Falcon ware</td>
</tr>
<tr>
<td>96 well tissue culture plates</td>
<td>Becton Dickinson, Cowley, Oxford.</td>
</tr>
<tr>
<td>(flat-bottomed)</td>
<td></td>
</tr>
<tr>
<td>1.2 mL cryotubes (Nunc)</td>
<td>Gibco, Paisley, Scotland, UK.</td>
</tr>
<tr>
<td>Filter units (250 mL capacity)</td>
<td>Merck Ltd., Magna Park, Lutterworth, Leicestershire.</td>
</tr>
</tbody>
</table>
Quill filling tubes

Avon Medical, Redditch, Worcestershire.

5, 10 and 25 m pipettes

Philip Harris, London.

**Nonsterile Disposable Plastics**

96 well round bottomed plastic flexible polyvinyl plates (300 µl/well)

Falcon ware, Becton Dickinson.

Pipette tips (Gilson)

Anachem, Luton,

96 well flat bottomed polystyrene plates for ELISAs

Costar (UK) (3590) High Wycombe, UK.

**Glassware**

**Disposable glassware**

Sterile 1 mL and 2 mL pipettes

British Drug House (BDH), Poole, UK.

Glass slides

Miles Lab. Inc. Naperville, Illinois, USA

Tissue culture chamber slides (Lab-Tek)
REAGENTS

**Chemicals**

- Acetaldehyde
- Acetic acid
- Agarose
- Aminoethylisothiouronium bromide hydrobromide (AET)
- Bovine serum albumin
- Calcium ionophore (Cal A23187)
- Calf intestinal alkaline phosphatase
- Cycloheximide
- Diphenylamine
dNTP
- Ethidium bromide
- Ethylenediaminetetraacetic acid (EDTA)
- $\text{H}_2\text{SO}_4$
- Histopaque
- Hydrocortisone
- KCl
- MgCl$_2$
- Na acetate
- N$^\alpha$-benzyloxycarbonyl-L-lysine thiobenzyl ester
- NaCl
- O-phenylene diamine (OPD)
- Poke-weed mitogen (PWM)
- 12-0-tetradecanoylphorbol 13-acetate (TPA)
- Tricarboxylic acid

**Source**

- Sigma Chemicals
- Poole, Dorset, UK.
- Rathburn Chemical
- Walkerburn, Scotland
- Promega,
- Southampton, UK.

Triton X-100

Tween 20
Chloroform
Ethanol
Fast Blue BB
Fast Red/Naphthol ASBl
Gelatin
Glutaraldehyde
Harris' Haematoxylin
Hydrogen peroxide
Preservative free Heparin
Proteinase K
SDS
Tris
Tris-HCl

Lymphoprep

\(^{51}\)Chromium
\(^{3}H\)-Thymidine
Na\(_2^{35}\)SO\(_4\)

**Buffers**

Phosphate buffered saline (PBS) solution was made from PBS tablets (Oxoid Ltd., Basingstoke, Hampshire, UK). PBS/Tween for use in ELISAs was made by adding 0.05% of Tween 20 to PBS solution.

Phosphate buffer, pH 8 was made by titrating 0.1M NaH\(_2\)PO\(_4\) with 0.1M Na\(_2\)HPO\(_4\).

Citrate/phosphate buffer was made using 0.1M citric acid, and titrating in the appropriate amount of 0.1M Na\(_2\)HPO\(_4\) to reach the required pH.
**Tissue culture media, serum and enzymes Source**

Phosphate buffered saline (PBS)
RPMI
Hank's Balanced Salt Solution (HBSS)
Iscove's Modified Dulbecco's Medium (IMDM)
Penicillin/Streptomycin
Trypsin/EDTA

Collagenase (*Clostridium histolyticum*, Type A)
Fibronectin

Collagenase (Clostridium histolyticum, Type A)

Fibronectin

Boehringer
Mannheim
Lewes,
Sussex.

Foetal calf serum (FCS) was obtained from Gibco Ltd. (Paisley, Scotland). It was first heat-inactivated at 56°C for 30 minutes, filtered using 2μm pore filter units, and aliquots were stored at -20°C. Batches were tested for their ability to support optimal cell growth *in vitro*.

**Cytokines**

Recombinant interferon-alpha (rInterferon-alpha) was obtained from Roche laboratories, Switzerland; interferon-gamma from Biogen Research Corporation, Cambridge, Massachusetts, USA. Interleukin 1-β (IL-1β) was obtained from Boehringer-Mannheim, Germany. Interleukin-4 (IL-4) and interleukin-6 (IL-6) were gifts from the National Institute for Biological Standards and Control, UK. Interleukin-2 (IL-2) was a gift from Eurocetus Corp., USA. Tumour necrosis factor alpha (TNF-α) was a gift of Immunex Corp., USA. Endothelial cell growth supplement (ECGS) was obtained from Sigma Chemicals, UK.
ANTIBODIES

Commercial antibodies

Leu-8 (anti-L-selectin), Leu 3 (anti-CD4) and Leu M5 (anti-CD11c) monoclonal antibodies (mAbs) were obtained from Becton-Dickinson, UK; anti-CD44 was obtained from The Binding Site, UK; TQ1 (anti-L-Selectin) mAb was obtained from Coulter Electronics, UK. Rabbit anti-mouse flourescein conjugated antibody (RAM-FITC), MHM24 (anti-CD11a), anti-HLA-DR, anti-CD19, anti-IgM and anti-von Willebrand factor (anti-vWf Ag) mAbs were obtained from DAKO Ltd., High Wycombe, UK. Anti-VLA-4, anti-ELAM-1 and anti-VCAM-1 mAbs were purchased from British Biotechnology, UK.
The following antibodies were gifts:

<table>
<thead>
<tr>
<th>Name</th>
<th>Specificity</th>
<th>Subclass</th>
<th>Reference</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAM 1.1</td>
<td>anti-L-selectin</td>
<td>IgG1</td>
<td>Tedder et al 1989</td>
<td>Dr. Tedder, Dana Farber Cancer Institute, USA;</td>
</tr>
<tr>
<td>15.2</td>
<td>Anti-ICAM-1 (CD54)</td>
<td>IgG1</td>
<td>Dransfield et al 1989</td>
<td>Dr. Nancy Hogg, Imperial Cancer Research Fund, London, UK.</td>
</tr>
<tr>
<td>UCHL-1</td>
<td>anti-CD45RO</td>
<td>IgG2a</td>
<td>In:Leucocyte Typing II</td>
<td>Prof. P Beverley, University College, London, UK.</td>
</tr>
<tr>
<td>UCHT-1</td>
<td>anti-CD3</td>
<td>IgG1</td>
<td>Reinherz et al. (1986)</td>
<td></td>
</tr>
<tr>
<td>UCHT-4</td>
<td>anti-CD8</td>
<td>IgG1</td>
<td>Springer-Verlag, New York.</td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>anti-bcl-2</td>
<td>IgG1</td>
<td>Pezzella et al 1990</td>
<td>Dr. DY Mason, John Radcliffe Hospital, Oxford, UK.</td>
</tr>
</tbody>
</table>

Table 2.1 Details of non-commercial monoclonal antibodies
2.2 PATIENTS

Patients with B-cell chronic lymphocytic leukaemia (CLL), diagnosed according to standard clinical and laboratory criteria (Catovsky & Foa, 1990) at Binet Stage A were entered into a nonrandomized clinical trial of interferon-alpha, supplied by Roche Products Ltd., Welwyn Garden City, U.K. All patients received 3 mega-units (3MU) by subcutaneous injection 3 times weekly. Patients were assessed on a weekly basis during the first month of therapy, monthly during the second and third months, and three monthly thereafter. Response criteria were defined as follows: Complete Remission (CR): disappearance of all palpable disease and restoration of all haematological parameters including bone marrow morphology to normal, Partial Haematological response (PHR): reduction of absolute lymphocyte counts by 50% or more of the pretreatment values.

2.3 GENERAL METHODS

Peripheral cell counts
Venous blood taken into EDTA was analysed on an automated cell counter (STAK-R, Coulter Electronics, Luton UK).

Cell separation.
Peripheral blood from patients or normal controls was collected into preservative free heparin and separated on Ficoll-Hypaque gradients. Mononuclear cells (MNC) were then washed three times in RPMI 1640 medium with 10% heat inactivated foetal calf serum, 200U/mL penicillin and 100mg/mL streptomycin (RPMI-
FCS). Leukaemic cells from CLL patients and B-cells from normal controls were further enriched by depletion of sheep red blood cell rosetting cells. Briefly, 10mL of aminoethylisothiouronium bromide hydrobromide (AET) solution (0.403g/10mL H₂O, pH 9.0 ) were added to 2 mL of saline washed sheep erythrocytes, the mixture was incubated for 30 minutes at 37°C, and washed four times in PBS before resuspending in RPMI-FCS. For rosetting, 300μL of 50% AET treated sheep erythrocytes were added to 2x10⁷ mononuclear cells, mixed well, centrifuged, and placed on ice for one hour. The bulk rosetted MNC were then separated on a further Ficoll-Hypaque gradient. Non-rosetting E-negative cells were collected from the interface and washed three times in RPMI-FCS. Greater than 95% of the E-negative cells from CLL patients, and 50-60% of the E-negative cells from normal controls were B cells, as assessed by reactivity with anti-CD19 mAb.

**Determination of cell surface antigen expression**

All antibodies were titrated out and used at optimal concentrations.

**Indirect immunofluorescence**

Cells were incubated with saturating amounts of antibody for 45 minutes at 4°C, washed three times with RPMI-FCS and then further incubated with RAM-FITC for 45 minutes at 4°C. After a further three washes in RPMI-FCS the cells were fixed in 3% formalin in PBS. Negative controls were stained with isotype-matched monoclonal antibody. Cells were analysed on an EPICS CD flow cytometer (Coulter Electronics, Luton, U.K.). Both antigen density, measured as mean cell fluorescence intensity on a linear
scale, and percentage positive cells (defined such that negative samples contained 5% positive cells) were determined.

**APAAP Method**

Cytocentrifuge preparations were sequentially incubated for 30 minutes with primary antibody, followed by rabbit anti-mouse Ig, then with the APAAP complex and finally with Fast Red/Naphthol ASBI (10mg Fast Red Tr salt, 1mL naphthol AS-MX, 50μL Levamisole in 0.2M Tris-HCl pH 8.1) as chromogen/substrate solution. The APAAP complex was made by incubating murine anti-alkaline phosphatase mAb (kindly provided by Dr. P. Beverley, University College, London) with calf intestinal alkaline phophatase overnight at 4°C. Slides were washed for 5 minutes in PBS (pH 7.2) between each incubation. The slides were counterstained with Harris' Haematoxylin, rinsed in distilled water, dried and mounted in Apathy's mountant.

**Anti-interferon antibody assay**

Serum samples were analysed for anti-interferon antibodies in the Roche Laboratories, Basle, Switzerland, using an enzyme immunoassay (Hennes et al 1987). Briefly, serum samples were incubated with interferon-alpha 2a covalently attached to plastic beads. Following this, the beads were washed and incubated with peroxidase labelled interferon-alpha 2a . Excess peroxidase conjugate was removed by washing and substrate (OPD/H2O2) added. The reaction was stopped using 1M H2SO4 and the optical density read at 492 nm on a microplate reader as above. The interferon-binding titre was expressed as interferon-binding units /mL (IBU/mL).
**Statistical methods**

Statistical analyses were performed using Student's t-test, and the StatView programme on the Apple Macintosh.
CHAPTER 3
HAEMATOLOGICAL RESPONSES TO INTERFERON-ALPHA IN PATIENTS WITH EARLY STAGE CLL

INTRODUCTION

The interferons were initially described as soluble substances which could interfere with viral replication in uninfected cells (Isaacs & Lindeman 1957). As the proteins were purified it became apparent that interferons had a wide variety of effects on cells, including antiproliferative effects, and were also able to modulate cell differentiation and immune function (De Maeyer & De Maeyer Guignard 1988). Demonstration of the anti-neoplastic effects of interferon excited interest in its role as a potential therapeutic agent for malignant diseases (Gresser 1972, Gresser & Tovey 1978). Difficulties with production and purification meant that initial trials were limited by the small amounts of human interferons available. With the advent of human interferon-alpha produced by lymphoblastoid cells stimulated with Sendai virus, and the subsequent availability of recombinant proteins, use of interferons as therapy in a variety of human cancers soon became widespread (Kirkwood & Ernstoff 1984, Krown 1986, Goldstein & Laszlo 1986). Early results were encouraging, and it soon became apparent that interferon-alpha was effective in a variety of haematological malignancies (Roth & Foon 1986, Jahiel & Krim 1987, Galvani & Cawley 1990, Heslop et al 1992). Of these, interferon-alpha had the most dramatic effect in hairy cell leukaemia.
Hairy cell leukaemia is a lymphoproliferative disorder of B-cell origin, in which the hairy cells infiltrate the blood, bone marrow and the reticulo-endothelial system (Cawley & Worman 1985, Golde et al 1986). Previous therapy had been based on splenectomy, but often only transient relief of symptoms was achieved, and refractory patients were difficult to manage. Following the initial report of the therapeutic efficacy of interferon-alpha in hairy cell leukaemia (Quesada et al 1984), trials have consistently shown responses in 75-90% of patients (Roth & Foon 1986, Jahiel & Krim 1987, Galvani & Cawley 1990, Heslop et al 1992).

Because of the successful treatment of hairy cell leukaemia with interferon-alpha, many groups were encouraged to use interferon-alpha in CLL. Early studies in advanced disease were disappointing (Foon et al 1985), but several studies have now shown that interferon-alpha can be effective therapy in patients with early stage CLL (O’Connell et al 1986, Pangalis et al 1988, Rozman et al 1988, Ziegler-Heitbrock et al 1989). In these studies most patients achieved a partial response but few gained complete response. These studies prompted the establishment in 1988 of an open non-randomised pilot study into the effects of low dose interferon-alpha 2a in early stage CLL, some of the results of which are presented in this chapter.
PATIENTS

The diagnosis of B-cell chronic lymphocytic leukaemia (CLL) was made according to standard clinical and laboratory criteria (Foon & Gale, 1990). Patients had a minimum absolute lymphocytosis of $10 \times 10^9$/L, less than 10% "atypical" and/or prolymphocytoid peripheral lymphocytes, and immunological markers compatible with the diagnosis. Table 3.1 shows the phenotype analysis of the 13 patients whose data are presented in this chapter. Previously untreated patients with disease at Binet stage A (see Chapter 1) were selected for entry into an open, non-randomized pilot study of recombinant interferon-alpha 2a (supplied by Roche Products Ltd., Welwyn Garden City, U.K). All patients received 3MU of interferon-alpha by subcutaneous injection 3 times weekly for a 2 year period. Patients were assessed on a weekly basis during the first month of therapy, monthly during the second and third months, and three monthly thereafter. Response criteria were defined as follows: Complete Remission (CR): disappearance of all palpable disease and restoration of all haematological parameters including bone marrow morphology to normal, Partial Haematological response (PHR): reduction of absolute lymphocyte counts by 50% or more of the pretreatment values.
RESULTS

**Phenotype analysis**

At the time of writing, 13 patients were available for analysis. Table 3.1 gives the results of phenotype analysis carried out on peripheral blood mononuclear cells in each of these patients. All patients had the typical immunological phenotype of CLL. Interferon-alpha therapy had no significant effect on the cell surface phenotype, as assessed at 3 months (Table 3.1).

**Peripheral cell counts**

Table 3.2 gives details concerning the age, sex, duration of therapy and haematological response of these 13 patients. All patients demonstrated a clinical response in terms of a fall in lymphocyte counts. Lymphocyte counts decreased from a median of $21.3 \times 10^9$ L (range 11.5 - 95) to a median nadir of $11.0 \times 10^9$ L (range 2.9 - 43.4 $\times 10^9$ L, p<.01, n=13). Figure 3.1 shows the timing and levels of nadir counts reached, for each of the 13 patients. The nadir of lymphocyte counts was reached at a variable time from the start of therapy; 7 patients reached their nadir counts within 3 months of starting treatment, while 2 patients took a year to do so.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Time</th>
<th>Lymphocyte count (X10^9/L)</th>
<th>%CD3</th>
<th>%CD5</th>
<th>%CD19</th>
<th>%kappa</th>
<th>%lambda</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL 1</td>
<td>pre</td>
<td>24.5</td>
<td>1</td>
<td>95</td>
<td>89</td>
<td>&lt;1</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>16.3</td>
<td>6</td>
<td>96</td>
<td>93</td>
<td>&lt;1</td>
<td>85</td>
</tr>
<tr>
<td>CLL 2</td>
<td>pre</td>
<td>95</td>
<td>5</td>
<td>99</td>
<td>91</td>
<td>90</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>60</td>
<td>2</td>
<td>99</td>
<td>96</td>
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</tr>
<tr>
<td>CLL 3</td>
<td>pre</td>
<td>16.8</td>
<td>10</td>
<td>10</td>
<td>90</td>
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<td>90</td>
</tr>
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<td>7.8</td>
<td>15</td>
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<td>84</td>
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<td>CLL 4</td>
<td>pre</td>
<td>21.3</td>
<td>11</td>
<td>94</td>
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<td>post</td>
<td>8.8</td>
<td>15</td>
<td>95</td>
<td>86</td>
<td>87</td>
<td>&lt;1</td>
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<td>CLL 5</td>
<td>pre</td>
<td>13.9</td>
<td>18</td>
<td>96</td>
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<td>14.5</td>
<td>14</td>
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<tr>
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<td>post</td>
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<td>17</td>
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<td>CLL 7</td>
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<tr>
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<td>65</td>
<td>ND</td>
<td>73</td>
<td>&lt;1</td>
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<td>ND</td>
</tr>
<tr>
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<td>post</td>
<td>17.9</td>
<td>4</td>
<td>95</td>
<td>93</td>
<td>ND</td>
<td>87</td>
</tr>
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<td>CLL 12</td>
<td>pre</td>
<td>25.6</td>
<td>15</td>
<td>91</td>
<td>86</td>
<td>85</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>11.5</td>
<td>14</td>
<td>96</td>
<td>88</td>
<td>87</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CLL 13</td>
<td>pre</td>
<td>93.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>post</td>
<td>50.9</td>
<td>5</td>
<td>98</td>
<td>90</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3.1. Phenotypic analysis of peripheral blood in CLL patients before and after (at 3 months) starting on interferon therapy. Peripheral blood mononuclear cells were separated as detailed in Chapter 2. Determination of surface phenotype was carried out by indirect immunofluorescence, and analysis by flow cytometry.

* 4 week values as anti-IFN antibodies detected at 3 months
** 8 week values as anti-IFN antibodies present at 3 months
***values at 80 weeks
<table>
<thead>
<tr>
<th>Patient no /age/sex</th>
<th>Lymphocyte counts (X10⁹/L)</th>
<th>Time of nadir (weeks)</th>
<th>Duration of continuous IFN therapy (months)</th>
<th>Response maintained during therapy?</th>
<th>Antibody status during period of follow up (titre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pretreatment</td>
<td>nadir</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLL 1/60/M</td>
<td>24.5</td>
<td>16.3</td>
<td>12</td>
<td>30</td>
<td>yes</td>
</tr>
<tr>
<td>CLL 2/67/F</td>
<td>95.0</td>
<td>21.2</td>
<td>52</td>
<td>24</td>
<td>partial/no</td>
</tr>
<tr>
<td>CLL 3/66/F</td>
<td>16.8</td>
<td>4.4</td>
<td>41</td>
<td>16</td>
<td>yes, but lost when IFN stopped</td>
</tr>
<tr>
<td>CLL 4/66/M</td>
<td>21.3</td>
<td>8.8</td>
<td>13</td>
<td>13</td>
<td>yes</td>
</tr>
<tr>
<td>CLL 5/63/M</td>
<td>13.9</td>
<td>5.4</td>
<td>3</td>
<td>24</td>
<td>no, lost at 11 weeks</td>
</tr>
<tr>
<td>CLL 6/60/M</td>
<td>14.5</td>
<td>10.5</td>
<td>2</td>
<td>8</td>
<td>yes, but lost when IFN stopped</td>
</tr>
<tr>
<td>CLL 7/77/F</td>
<td>73.0</td>
<td>43.4</td>
<td>19</td>
<td>6.5</td>
<td>yes</td>
</tr>
<tr>
<td>CLL 8/45/F</td>
<td>36.4</td>
<td>12.9</td>
<td>6</td>
<td>6</td>
<td>no, lost at 6 months</td>
</tr>
<tr>
<td>CLL 9/54/M</td>
<td>12.9</td>
<td>2.9</td>
<td>65</td>
<td>44</td>
<td>yes</td>
</tr>
<tr>
<td>CLL 10/66/F</td>
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<td>4.0</td>
<td>4</td>
<td>5</td>
<td>no</td>
</tr>
<tr>
<td>CLL 11/70/F</td>
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<td>14.7</td>
<td>6</td>
<td>12</td>
<td>no</td>
</tr>
<tr>
<td>CLL 12/67/M</td>
<td>25.6</td>
<td>11.5</td>
<td>34</td>
<td>6</td>
<td>no</td>
</tr>
<tr>
<td>CLL 13/58/M</td>
<td>93.1</td>
<td>11.4</td>
<td>34</td>
<td>24</td>
<td>no, lost at 1 year</td>
</tr>
</tbody>
</table>

Table 3.2. Clinical and haematological data in CLL patients who received interferon therapy
Figure 3.1. Lymphocyte counts in 13 patients with early stage CLL receiving interferon-alpha therapy. Pretreatment, and nadir counts are shown for each patient.
All haematological parameters (Haemoglobin, lymphocyte, neutrophil and platelet counts) were analysed at 4 weeks, 3 months and 6 months after starting therapy, and these results are given for the 4 week and 3 month time points in Table 3.3. The fall in lymphocyte counts is most marked at 4 weeks, and neutrophil counts also demonstrate a significant fall at this time. The rise in median lymphocyte count between 4 weeks and 3 months reflects the loss of response in 6 patients (see Table 3.2). Of these 6 patients, 4 had developed anti-IFN antibodies (see below). 2 patients lost response while on therapy, but had no measurable anti-interferon antibodies.

**Development of anti-interferon antibodies**

4 patients developed anti-interferon antibodies, as assayed by ELISA (see Table 3.1). 3 of these patients developed high titre antibodies at 11-13 weeks, and had been antibody negative previously. The fourth patient was found to have antibodies at time of first testing at 17 months. The rise in antibody titre is accompanied by a corresponding rise in the lymphocyte count, indicating loss of clinical response. This is shown for each of the 3 patients who had serial measurements (Figures 3.2 a-c). Antibody titres declined after an initial rise to a peak of 5000-15000 IBU at 11-24 weeks. In 1 patient (Figure 3.2a), the reduction in antibody titre was accompanied by a corresponding fall in the lymphocyte count.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>at 4 weeks</th>
<th>at 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hb (g/dL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pretreatment</td>
<td>median</td>
<td>median</td>
</tr>
<tr>
<td></td>
<td>range</td>
<td>range</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td>12.3 - 14.6</td>
<td>12.3 - 14.6</td>
</tr>
<tr>
<td></td>
<td>(12) NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>13.4</td>
<td>12.1 - 14.5</td>
</tr>
<tr>
<td></td>
<td>(9) NS</td>
<td></td>
</tr>
<tr>
<td><strong>lymphocytes</strong></td>
<td>median</td>
<td>median</td>
</tr>
<tr>
<td>(10^9/L)</td>
<td>range</td>
<td>range</td>
</tr>
<tr>
<td></td>
<td>21.3</td>
<td>11.8</td>
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<tr>
<td></td>
<td>11.5 - 95.0</td>
<td>4.0 - 65.0</td>
</tr>
<tr>
<td></td>
<td>(13) **</td>
<td>(12)</td>
</tr>
<tr>
<td></td>
<td>16.7</td>
<td>4.2 - 74.5</td>
</tr>
<tr>
<td></td>
<td>(9) *</td>
<td></td>
</tr>
<tr>
<td><strong>neutrophils</strong></td>
<td>median</td>
<td>median</td>
</tr>
<tr>
<td>(10^9/L)</td>
<td>range</td>
<td>range</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>3.4</td>
</tr>
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<td></td>
<td>2.9 - 8.9</td>
<td>1.3 - 5.7</td>
</tr>
<tr>
<td></td>
<td>(13) **</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>1.7 - 9.1</td>
</tr>
<tr>
<td></td>
<td>(9) NS</td>
<td></td>
</tr>
<tr>
<td><strong>platelets</strong></td>
<td>median</td>
<td>median</td>
</tr>
<tr>
<td>(10^9/L)</td>
<td>range</td>
<td>range</td>
</tr>
<tr>
<td></td>
<td>205</td>
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<td>169</td>
<td>114 - 213</td>
</tr>
<tr>
<td></td>
<td>(9) NS</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3. Medians and ranges of haematological parameters of all 13 patients, prior to starting therapy, and at 4 weeks and 3 months afterwards. Figures in brackets give the number of patients evaluable at that time point. * = p < 0.05, ** = p < 0.01, NS = nonsignificant.
Figure 3.2. Development of anti-interferon antibodies in 3 patients, and correlation with lymphocyte count. (a) patient CLL 5, (b) patient CLL 10, (c) patient CLL 12 (overleaf).
Figure 3.2(c). Anti-interferon antibody titre and lymphocyte count in patient CLL 12.
DISCUSSION

The achievement of partial responses in patients with early stage CLL receiving interferon-alpha is in accord with previous studies (Rozman et al 1988, Pangalis & Griva 1988, Ziegler-Heitbrock et al 1989). In the group of patients presented here, 6 out of 13 (46%) lost response while on therapy. In 4 of these patients, high titre anti-interferon antibodies were detected at the time when clinical response was lost, and the lymphocyte count rose in parallel with the titre of antibody. The development of anti-interferon antibodies, and the correlation with loss of clinical response has been reported in other conditions treated with interferon alpha, such as hairy cell leukaemia, chronic hepatitis B infection and chronic myeloid leukaemia (Steiss et al 1988, von Wussow et al 1987, Porres et al 1989, Freund et al 1989). The loss of such antibodies with continued treatment has also been reported in one study of patients with hairy cell leukaemia (Steiss et al 1991). In the study presented in this thesis, the loss of anti-interferon antibodies was associated with recovery of clinical response in 1 patient. The clinical significance of the development of these antibodies remains to be fully elucidated, but patients with anti-interferon antibodies have been shown to regain the response if the recombinant interferon is replaced by a non-recombinant 'natural' interferon which contains all the subtypes of alpha interferon (Steiss et al 1991).

Although interferon-alpha therapy is able to produce clinical responses in CLL it is not clear whether this will have any effect on long term survival. This is a problem in a disease in which median survival may be as long as ten years. Although
treatment with chlorambucil in Binet stage A CLL has not been shown to improve long term survival, despite producing clinical remissions, it is not possible to extend these arguments to interferon-alpha therapy. As a modulator of immune functions, interferon-alpha has the potential to alter the immune status of the patient and possibly to alter the course of the disease. However, although interferon-alpha therapy is well tolerated, the problems associated with long term therapy remain formidable. Apart from the unknown immunological and pathological consequences of long term interferon-alpha therapy, the financial costs, the problems of long term patient compliance, and the advent of new therapeutic agents make long term trials unlikely. Furthermore, although interferon-alpha appears most effective in stage A patients, the desirability or legitimacy of treating patients in early stage disease remains controversial. If median survival of stage A patients is greater than ten years, perhaps our energies are best concentrated on establishing diagnostic criteria to distinguish those patients whose disease will run an indolent course for many years without treatment, from those who would benefit from clinical intervention at an early stage, and designing therapy appropriately. This will undoubtedly depend on increased understanding of the biology and natural history of the disease.

Although interferon-alpha produced some good partial responses, no complete responses were seen in this study, and complete responses have been rare in other studies. Therefore it seems unlikely that interferon-alpha will be adopted as the sole agent of choice for the treatment of CLL, particularly since the new purine analogues such as fludarabine will shortly be licensed for use in the treatment of CLL. However, there is still much
interest in interferon-alpha as a therapeutic agent in combination with other therapeutic modalities, such as chemotherapy, radiotherapy or bone marrow transplantation. Rational design of these therapeutic options relies on our understanding of the mechanism of action of interferon-alpha in CLL. For example, if interferon-alpha is producing the effect by a cytostatic or non-proliferative effect, it may be necessary to choose cytotoxic drugs which are active in interphase or which do not rely on the cell cycle to be effective. If, on the other hand, interferon-alpha is activating immune effector mechanisms which are cytotoxic for the CLL cells, immunosuppressive therapy with cytotoxic drugs or radiation may be inappropriate, and it may be more relevant to treat in association with another biological response modifier such as IL-2.

The rest of this thesis, therefore, examines possible mechanisms whereby interferon-alpha is able to reduce peripheral lymphocyte counts in these patients.
CHAPTER 4
T-CELL ACTIVATION DURING INTERFERON-ALPHA THERAPY IN PATIENTS WITH EARLY STAGE CLL

INTRODUCTION

The anti-tumour effects of interferons are mediated by a number of different mechanisms, some or all of which may be involved in the response in a particular malignancy. These action of interferons can result from direct effects on tumour cells, for example, to inhibit proliferation, induce differentiation, alter gene (including oncogene) expression, and to alter expression of cell surface antigens, or may occur indirectly, via the activation of various effector mechanisms. The effects of interferon on the cytotoxic capability of the effector cells of the immune system, i.e. natural killer (NK) cells, T-cells and monocyte/macrophages, may contribute to the anti-tumour activity of interferons. Interestingly, the function of these effector cell populations has been found to be depressed in patients with malignant disease, including CLL (Zaknoen & Kay 1990).

Natural killer cells are able to lyse syngeneic and allogeneic tumour cells, and in animal models transfer of NK cells or their precursors can inhibit primary and metastatic tumour growth in vivo (reviewed in Trinchieri 1989). Studies of NK activity in humans have not established a clear relationship between NK activity and the incidence of malignancy and disease progression (Trinchieri 1989, Robertson & Ritz 1990). However, patients with advanced malignant disease often show depressed NK cell activity, and low NK cell activity appears to predispose to
lymphoproliferative disorders (Oshimi et al 1983, Lotsova et al 1987). In CLL, absolute numbers of natural killer cells may be increased, but NK cell activity, both spontaneous and antibody dependent cytotoxicity, is decreased. I and others have previously shown that IL-2 and interferon-alpha can correct these abnormalities in vitro (Platsoucas et al 1980, Santiago-Schwarz et al 1990, Jewell et al 1992). Generation of lymphokine activated killer (LAK) cell activity is also defective. CLL cells appear resistant to both NK cell and LAK cell activity in most patients (Foa et al 1990, Jewell et al 1992). Although NK cell activity can be enhanced by interferon therefore, it seems unlikely that this cytotoxic effector cell is responsible for the fall in the numbers of malignant B cells in the peripheral blood of patients with CLL on interferon-alpha therapy. However, the defects in NK cell activity are particularly interesting in view of the extensive data that support a role for NK cells in the regulation of B-cells and immunoglobulin production, and the association with the pathophysiology of CLL and the immune defects in this disease (Abruzzo & Rowley 1983, James & Ritchie 1984, Commes et al 1990).

Absolute numbers of T-cells are increased in CLL, but these cells appear to be defective in a number of functions. Defects of T-cell colony growth, increases in suppressor activity, decreases in helper activity, impaired responses to mitogens, abnormal mixed lymphocyte reactivity, production of IL-2 have been reported (Foon & Gale 1988, Freedman 1990, Zaknoen & Kay 1990). Interferon-alpha can enhance the specific cytotoxicity of sensitized lymphocytes against allogeneic tumour cells (Lindahl et al 1972, Heron et al 1976). Furthermore, interferon-alpha can
induce T-cell receptor gene rearrangements and the acquisition of antigen specific cytotoxic activity in T cell clones (Chen et al 1986a, Chen et al 1986b). Interferon-alpha therapy in CLL may therefore produce a therapeutic effect by enhancing or inducing an antigen specific T-cell response against the malignant clone. This chapter examines the effect of interferon-alpha therapy on in vivo parameters of T-cell activation in patients with early stage CLL.
PATIENTS

8 out of the group of 13 patients, studied in Chapter 3, were examined for the following *in vivo* markers of T cell phenotype and function.

SPECIAL METHODS

**Soluble IL-2 receptor assay**
Levels of soluble IL-2 receptor (sIL-2R) were determined in an enzyme immunoassay (T-cell Diagnostics, USA). Serum samples were added to wells in a microtitre plate coated with a monoclonal antibody specific for IL-2R, followed immediately by a second monoclonal antibody specific for a distinct epitope on IL-2R conjugated to horseradish peroxidase. Following incubation for two and a half hours at room temperature on a rotator, the plate was washed, and freshly prepared chromogen substrate was added (O-phenylenediamine). After 30 minutes incubation at room temperature, the reaction was stopped by adding 2N sulphuric acid, and the optical density measured at 490nm with wavelength correction at 570nm, on an MR700 microplate reader (Dynatech Laboratories, UK). The concentration of sIL-2R was determined by reference to a standard curve.

**CD4 and CD8 assay**
Levels of soluble CD4 and CD8 were determined in the same manner as for the sIL2R, using antibodies specific for CD4 and CD8 respectively.
**Serine Esterase**

The serine esterase enzymes are a group of proteins found in the cytolytic granules of T and NK cells, which have been implicated in the cytotoxic activity of these cells. Demonstration of serine esterase at the cellular level is a novel technique which enables simultaneous estimation of cytotoxic potential and phenotype, and levels of serine esterase correlate well with cytotoxic assays (Wagner *et al* 1989). Cytocentrifuge preparations were air dried and fixed in 1% paraformaldehyde for 30 seconds. The slides were then transferred into the substrate/chromogen solution (2x10⁻⁴ M Nα-benzoyloxy carbonyl-L-lysine thiobenzyl ester and 0.16mg/mL Fast Blue BB salt in 0.2M Tris-HCl, pH 8.1, prepared immediately before use). After 15 minutes incubation at 37°C the slides were washed in PBS (pH7.2) and further stained using the alkaline phosphatase anti-alkaline phosphatase (APAAP) method described in Chapter 2.
RESULTS

Haematological responses in this subgroup of patients were similar to those described for the group as a whole in Chapter 3, and are given in detail in the Results section in Chapter 5.

**T cell numbers in CLL**

Absolute numbers of CD3 positive lymphocytes in this group of patients prior to treatment was 2.5±1.2X10⁹/L (mean±SD, median 2.3, range 1.0-4.7X10⁹/L, n=8), normal range 2.9±1.2X10⁹/L. Interferon therapy had no effect on the numbers of circulating CD3 positive cells when assessed at 4 weeks (2.6±1.5X10⁹/L, median 2.1, range 1.2-5.1X10⁹/L). At 12 weeks, however, there was a significant reduction in the numbers of CD3 positive cells (1.2±0.5X10⁹/L, median 1.2, range 0.5-1.9X10⁹/L, n=8, p<0.05). Absolute numbers of CD4 positive lymphocytes prior to treatment were 1.6±0.8X10⁹/L (mean±SD, median 1.3, range 1.0-3.3X10⁹/L, n=8), normal range 2.0±0.2X10⁹/L. Absolute numbers of CD8 positive lymphocytes prior to treatment were 1.1±0.9X10⁹/L (mean±SD, median 0.7, range 0.5-2.8X10⁹/L, n=8), normal range 1.0±0.1X10⁹/L. Interferon-alpha had no significant effect on numbers of CD4 positive and CD8 positive cells. Figures 4.1 (a-c) show the absolute numbers of CD3 positive, CD4 positive and CD8 positive cells in this group of patients, before, and at 4 and 12 weeks after starting on interferon-alpha therapy. CD4/CD8 ratios prior to treatment were 2.7±2.4, and at 12 weeks were 3.2±1.2 (n=5, NS).
Figure 4.1. Absolute numbers of (a) CD3 positive, (b) CD4 positive and (c) CD8 positive cells in CLL patients prior to, and 4 and 12 weeks after starting on interferon therapy. Figures (b) and (c) are shown overleaf.
Figure 4.1. Absolute numbers of (b) CD4 and (c) CD8 positive cells in patients with CLL, and the effect of interferon therapy.
**Percentage of serine esterase positive cells**

The percentage of CD3 positive cells which stained for serine esterase was 32±17 (median 27, range 9-59), normal range 25.1±3.3. The percentage of CD4 cells which stained positive for serine esterase was 18±18 (median 14, range 0-49), normal range 0.5±0.4, and of CD8 cells was 59±22 (median 48, range 41-84), normal range 32.4±3.0. None of these percentages changed significantly on interferon-alpha therapy, as shown in Figure 4.2 (a-c).

**Soluble IL-2 receptor levels**

Pretreatment soluble IL-2 receptor (sIL-2r) levels were 1378±960 U/mL (median 1070 U/mL, range 477-3536 U/mL). Levels in normal control subjects using this assay are 573±173 U/mL (n=50). Untreated early stage CLL patients had elevated sIL-2r levels. Interferon therapy had no significant effect on circulating sIL-2r levels (Figure 4.3). After 4 weeks of treatment, sIL-2r concentrations in these patients were 1412±670 U/mL (median 1460 U/mL, range 691-2458 U/mL).

**Soluble CD4 and CD8 levels**

Soluble CD4 levels in the same 8 patients prior to commencing on interferon therapy were 51±28 U/mL (median 40 U/mL, range 24-107 U/mL, normal range 33±15 U/mL). Soluble CD8 levels were 532±287 U/mL (median 431 U/mL, range 375-1115 U/mL, normal range using this assay is 336±99 U/mL). Interferon-alpha had no effect on either sCD4 or sCD8 levels, measured at 4 and 12 weeks after starting treatment (Figures 4.4 and 4.5).
Figure 4.2(a) Percentage of CD3 positive cells expressing serine esterase in CLL patients. Serine esterase expression, determined as outlined in *Special Methods*, was assessed prior to, and at 4 and 12 weeks after starting interferon-alpha therapy.
Figure 4.2 Effect of interferon therapy on the percentage of (b) CD4 positive, and (c) CD8 positive, cells expressing serine esterase in CLL.
Figure 4.3. Serum levels of soluble IL-2 receptor (sIL-2R) in CLL patients prior to, and at 4 and 12 weeks after starting on interferon-alpha.
Figure 4.4. Serum levels of soluble CD4 in CLL patients prior to, and at 4 and 12 weeks after starting interferon-alpha therapy.

Figure 4.5. Serum levels of soluble CD8 in CLL patients prior to, and at 4 and 12 weeks after starting interferon-alpha therapy.
DISCUSSION

The effects of interferon-alpha therapy on the parameters of T-cell activation measured here are particularly interesting when compared to the effects in another chronic B-cell malignancy, hairy cell leukaemia (HCL). HCL is highly responsive to interferon-alpha therapy (Quesada et al 1984, Worman CP et al 1985), and may be associated with T-cell dysfunction (Zaknoen & Kay 1990, Kluin-Nelemans 1991). Patients with hairy cell leukaemia have normal numbers of CD3 positive cells in remission or in quiescent disease, but in active disease there may be a fall in the absolute numbers of CD3 positive cells (Cawley et al 1984, Lauria et al 1984). In contrast, absolute numbers of CD3 positive cells in CLL are often normal in patients with early stage disease, as seen in this study, but increase in advanced disease and may correlate with disease stage (Zaknoen & Kay 1990). Absolute numbers of CD4 and CD8 positive cells are again normal in HCL in remission or quiescent disease. However, in active disease levels of CD4 cells are decreased, and levels of CD8 cells are increased leading to a decreased CD4/CD8 ratio (Cawley et al 1984, Lauria et al 1984). In CLL, absolute numbers of CD4 and CD8 positive cells may be normal in early stage disease, as shown in this chapter, or slightly increased. In advanced disease, absolute numbers of both CD4 and CD8 cells are increased, but the CD4/CD8 ratio is again decreased, due to relatively greater increase in CD8 positive cells (Zaknoen & Kay 1990). The responses to interferon-alpha therapy also show marked contrasts and similarities. Interferon-alpha therapy in HCL patients with advanced disease leads to an increase in T-cell
counts and an increase in the CD4/CD8 ratio (Foa et al. 1987). In CLL, interferon-alpha therapy results in a reduction in the total number of T-cells in some cases and may also decrease the CD4/CD8 ratio (O'Connell et al. 1986, Ziegler-Heitbrock et al. 1989). Therefore, in HCL and CLL interferon-alpha therapy appears to have opposite effects on the absolute numbers of T-cells, but which both tend to a normalization of the T-cell count and CD4/CD8 ratio.

When the numbers of those T-cells that are serine esterase positive are compared, another interesting contrast becomes apparent. In HCL, the numbers of serine esterase positive T-cells are normal in both the CD3 and CD8 populations and the levels of CD4 serine esterase positive cells is almost zero, as in normal populations. When the HCL patients are treated with interferon-alpha, the numbers of T-cells that are serine esterase positive increases (Wagner et al. 1989). Interestingly, the T cells in CLL patients who are not on therapy already show increased serine esterase positivity, and interferon-alpha therapy does not appear to alter these numbers. These results suggest that the T cells in CLL are already functionally activated and primed for cytotoxicity. Whether the T cells are specific for antigens on the malignant B cell clone, for autoantigens or determinants on infectious agents remains to be elucidated.

The levels of soluble IL2- receptor (sIL-2R) are raised in the serum of patients with HCL (Steis et al. 1988) and CLL (Semenzato et al. 1987), and although there is still some controversy about the origin of the sIL-2R it is likely that it is a product of the malignant cells in both diseases. In HCL, treatment with interferon-alpha leads to a reduction in the levels of sIL-2R
in those patients that respond to treatment, but remains above the normal range (Steis et al 1988, Ho et al 1990). In this study, levels of sIL-2R were raised and did not change on interferon-alpha therapy. This may reflect the large bulk of leukaemic cells remaining even after achieving partial remission in these patients.

Levels of soluble CD8 (sCD8) are also reported to be high in HCL and to decrease on interferon-alpha therapy (Ho et al 1990). It is not clear how this data is best interpreted. The authors suggest that the high levels of sCD8 reflect increased activation of suppressor/cytotoxic T-cells and the decrease on interferon-alpha therapy may be due to a reduction of suppressor/cytotoxic activity. However this interpretation seems at odds with the numbers of T-cells that express the serine esterase before and after interferon-therapy, as discussed above (Wagner et al 1989).

In the work presented in this chapter, it appears that the levels of both sCD8 and soluble CD4 (sCD4) are raised, and that levels do not change on interferon-alpha therapy. This is consistent with the data on serine esterase expression. It appears that interferon-alpha therapy may be having similar results in HCL and CLL via different mechanisms.

The data are consistent with the hypothesis that the T-cells are already activated in CLL, and that this does not change on interferon-alpha therapy. The apparent disparity between the suggestion that the T-cells are activated, and the widely reported defects in functional activity of T-cells in CLL, may reflect functional diversity of the cells. If a large proportion of the CD4 positive cells, for example, are committed to be cytotoxic cells as shown by their acquisition of the serine esterase, then it is
feasible that they will not be able to help in antibody production by B-cells or to undergo normal colony growth. While there is evidence for the presence of clonal populations of T-cells in CLL (Wen et al 1990), it is not clear whether these cells are specific for antigens on the malignant B cell clone, for autoantigens or determinants on infectious agents. Furthermore, if the T-cells are specific for the malignant B-cells, it is unclear why the anti-tumour response is not able to cure the disease. This may be related to the fact that the CLL cells are resistant to cell-mediated cytotoxicity (Foa et al 1990, Jewell et al 1992). It has also been suggested that the high levels of sIL-2R may result in sequestration of IL-2 and lead to defective T-cell responses.

In conclusion, these data fail to provide evidence that the effects of interferon-alpha therapy are due to T-cell activation in vivo.

Although the data presented here are consistent with T cell activation in these patients, it would be necessary to demonstrate increased expression of IL-2 receptor on the peripheral blood T cells in order to confirm this. Unfortunately it was not possible to do these studies, as the T cells from these patients were not available.
CHAPTER 5
MONONUCLEAR CELL ACTIVATION DURING INTERFERON-ALPHA THERAPY IN CLL

INTRODUCTION

Mononuclear phagocytes may be important in host defense against tumour growth and metastasis. Murine macrophages exhibit anti-tumour cytotoxicity when stimulated by cytokines, and cultured human monocytes display enhanced tumour cell killing in the presence of growth factors and other cytokines, including interferons (Grabstein et al 1986, Philip et al 1988, Crawford et al 1987). Monoclonal antibodies against tumour cells direct and enhance macrophage cytotoxicity in both human and murine systems (Adams et al 1984, Steplewski et al 1983).

Mononuclear phagocytes express the receptors for both interferon α/β and interferon γ (Finbloom et al, 1985, Celada et al, 1984), and interferon-alpha has been shown to regulate many of the immune functions of these cells. Interferon-alpha increases Fc receptor expression (Vogel et al 1983) and enhances both Fc-mediated, and nonreceptor-mediated phagocytosis by human and murine monocytes and macrophages in vitro and in vivo (Huang et al 1971, Donahue & Huang, 1976). Interferon-alpha also augments macrophage tumoricidal activity in vitro and in vivo in both murine (Gresser & Bourali 1970, Blasi et al 1984) and human (Dean & Virelizier 1983, Webb et al 1989) systems.

Activated mononuclear cells produce several cytokines and soluble mediators, one of which is macrophage colony-stimulating factor (M-CSF). M-CSF plays an important part in the
maturation and activation of monocytes/macrophages (Rettenmeir et al 1989), including the enhancement of antitumour cytoxicity (Nakoinz et al 1988, Suzu et al 1989). The activation of mononuclear cells during therapy with interferon-alpha may be associated with release of cytokines such as M-CSF, which could in turn operate in an autocrine/paracrine fashion to influence host immune and anti-tumour responses.

Neopterin is a pyrazino-pyrimidine compound which is an intermediate product in the biosynthesis of biopterin. It is released by peripheral blood mononuclear cells in response to stimulation with T cell derived lymphokines, including interferon-alpha and gamma-interferon in vitro (Huber et al 1984, Henderson et al 1991). Serum neopterin levels are elevated in a variety of infective and inflammatory conditions, including the acquired immunedeficiency syndrome and in allograft rejection, and are thus a useful marker of immune response activation (Fuchs et al 1988).

This chapter examines the effect of interferon-alpha therapy on circulating levels of M-CSF, and neopterin, in order to determine the possible role of mononuclear phagocytes in mediating clinical responses to interferon-alpha in patients with early stage CLL.
PATIENTS

Eight patients with Binet stage A CLL were studied. These patients were the same as those studied in Chapter 4. Patients received recombinant human interferon-alpha 2a (rhlnterferon-alpha2a, Roche products Ltd., U.K.) by subcutaneous injection, 3 MU 3 times a week, as outlined in Chapter 3. Venous blood samples were obtained prior to starting interferon therapy, and at various times afterwards. Sera was separated immediately, frozen and stored at -20°C, and thawed only once for use in M-CSF and neopterin assays.

SPECIAL METHODS

M-CSF Assay
Serum M-CSF concentrations were determined using an enzyme-linked immunosorbent assay employing antibodies raised human M-CSF purified from urine (hM-CSF), which has a specific activity of 1.5 X 10^8 U/mg (one unit being defined as the amount of hM-CSF needed to form a colony as determined by a mouse colony-forming assay, Hanamura et al 1988). Antibodies were prepared and purified as previously described (Hanamura et al 1988), and were kindly provided by Alpha Therapeutics, Japan. 96-well flat bottomed, polystyrene plates (Costar 3590) were coated with 100 μL of coating solution (consisting of horse anti-hM-CSF immunoglobulin fraction, diluted in phosphate buffered saline containing 0.02% sodium azide to an absorbance at 280 nm of 0.1) for 24 hours at 4°C. The plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS/Tween), and wells were
filled with 300 μL of blocking solution (10% immobilized normal horse serum, 0.25% BSA, 0.02% sodium azide in PBS) to block nonspecific binding of other proteins. The coated plate filled with blocking solution was sealed and stored at 4°C until use (within 1 month).

Prior to use, contents of each plate were discarded, and the plates washed 3 times with PBS/Tween. Test samples and hM-CSF standards, diluted 1 in 10 with dilution buffer (0.25% BSA, 0.3% gelatin, 0.02% sodium azide in phosphate buffered saline) were added to wells in duplicate, and incubated overnight at 4°C. Following 3 washes with PBS/Tween, rabbit polyvalent anti-hM-CSF antibody (100 μL/well) was added, and incubation continued for 2 hours at 37°C. The concentration of the rabbit anti-hM-CSF immunoglobulin used in this step was adjusted by diluting with blocking solution so that the rabbit protein had an absorbance at 280 nm of 0.5. After another 3 washes, horseradish peroxidase-conjugated goat IgG against rabbit IgG (0.005 to 0.05 μg/mL, Alpha Therapeutics) was added (100 μL/well) and left for 2 hours at room temperature. The wells were washed 5 times before addition of 100 μL of susbstrate (OPD, 0.8 mg/mL, in 0.1M citrate buffer, pH 5 with 0.015% H₂O₂ ) to each well. After 30 minutes in the dark at room temperature, the reaction was stopped with 2N H₂SO₄. The colour was read on an automatic microplate reader (Dynatech Laboratories), using dual beam wavelengths of 490 nm and 690 nm. The concentration of M-CSF was calculated from the corresponding standard curve.

The linear region of the standard curve was adjusted by varying the concentration of the conjugated antibody (Hanamura et al. 1988), thus giving a range of this assay from 10-2000
U/mL, i.e. the lower limit of detection was 100 U/mL or 660 pg/mL of M-CSF in a serum sample. The inter-assay variability was 9% and intra-assay variability was 5%. All antibodies were obtained from Alpha Therapeutics, Green Cross Corporation, Osaka, Japan.

**Neopterin Assay**

Serum neopterin levels were measured by radioimmunoassay (IMMU test Neopterin, Henning Berlin Gmbh, Germany, Honlinger et al 1989). A known amount of an $^{125}$I-neopterin tracer was added to standards and serum samples, followed by anti-neopterin antibody (sheep) pre-precipitated by anti-sheep IgG antibody (donkey). Following a one hour incubation in the dark at room temperature, tubes were then centrifuged to precipitate antigen-antibody complexes, and the precipitate-associated radioactivity counted using a gamma counter. Serum neopterin levels were calculated from the percentage drop in $^{125}$I-binding when compared with control.
RESULTS

Haematological responses in patients studied

Haematological responses in this subgroup of patients were similar to those described for the group as a whole, in Chapter 3. Prior to starting on interferon-alpha therapy, the lymphocyte count in these 8 patients was 33.8±31.0 X 10^9/L (mean±SD, median 17.5, range 9.2-95X10^9/L). All 8 patients showed haematological responses on interferon-alpha therapy; the nadir of the lymphocyte counts, achieved after 2-36 weeks of therapy, was 14.1±7.0X10^9/L (median 13.9, range 5.4-25.8X10^9/L, p<0.05). Responses were variable; 5 patients had a reduction of more than 50% in the absolute peripheral blood lymphocyte count, and the other 3 had a reduction of more than 25%. None achieved complete remission. Table 5.1 summarizes the data in these patients. The absolute lymphocyte counts fell to 61.7±19.5% of the initial levels by week 2 (p<0.01 by Student's t-test, p<0.05 by Wilcoxon signed-rank test), 68.5±19.4% by week 12 (p<0.05), and, in the 6 patients who did not develop anti-IFN antibodies, to 71.3±21.3% by week 24 (p<0.05, n=6).

Circulating M-CSF levels rose on interferon therapy

M-CSF levels in patients with untreated early stage CLL were 455±183 U/mL (median 520, range 160-610 U/mL, n=8). These levels fall within the normal range (540±110 U/mL) reported for this assay (Hanamura et al, 1989). Following initiation of interferon therapy, M-CSF levels rose to 686±110 U/mL (median 660, range 240 - 1110 U/mL) by week 2 (p<0.05), 924±248 U/mL (median 810, range 720 - 1410 U/mL) by week 12 (p<0.01, n=8),
and remained significantly elevated at week 24 (Table 5.1). All patients showed a rise in circulating M-CSF levels. Figure 5.1 shows the change in M-CSF concentrations in each patient after 2-4 weeks of treatment. Both the reduction in lymphocyte counts, and the increase in M-CSF levels were evident by week 2. The proportionate rise in M-CSF levels varied among the patients (maximum increases ranged from 132% to 530% of baseline levels). In 3 patients who had 4 or more serial measurements over a year from initiation of interferon therapy, there was a close correlation between the percentage reduction in peripheral counts, and the percentage increase in M-CSF levels (correlation coefficients for these patients were $r=0.8$, $r=0.8$, and $r=0.6$).

**Serum neopterin levels increased with interferon-alpha therapy**

In untreated early stage CLL, serum neopterin levels were 5.2±2.1 nmol/l (range 3.0-7.5 nmol/L, n=8). These concentrations fall within the reported normal range of serum neopterin (5.2±2.3 nmol/l, Honlinger et al 1989). Interferon-alpha therapy produced a rise in neopterin levels to 16.0±8.8 nmol/L by week 2 ($p<.01$). Neopterin levels remained elevated at 16.6±8.6 nmol/L at week 12 ($p<.01$), and at 14.6±9.6 nmol/L at week 24 ($p<.05$, n=4, Table 5.1). When followed serially in patients, the proportionate rise in neopterin levels was not correlated with the reduction in cell counts unlike the case with M-CSF levels.
<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Lymphocyte count (% of day 0)</th>
<th>M-CSF (U/mL)</th>
<th>Neopterin (nmol/L)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>100</td>
<td>455 ± 183</td>
<td>5.2 ± 2.1</td>
</tr>
<tr>
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</tr>
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<td>68.5 ± 19.4 *</td>
<td>924 ± 248 **</td>
<td>16.6 ± 8.6 **</td>
</tr>
<tr>
<td>24</td>
<td>71.3 ± 21.3 *</td>
<td>915 ± 390 *</td>
<td>14.6 ± 9.6 *</td>
</tr>
</tbody>
</table>

Table 5.1. Lymphocyte counts (expressed as a percentage of pretreatment levels) and serum M-CSF and neopterin concentrations in patients prior to, and at 2, 12, and 24 weeks after commencing on interferon-alpha therapy. Data are mean ± SD of 8 patients.

* p < 0.05, ** p < 0.01
Figure 5.1. Effect of interferon-alpha therapy on serum M-CSF levels. Serum M-CSF levels were determined by ELISA as detailed in Special Methods, before and at 2 weeks after starting on interferon. Data for each of the 8 patients studied is given.
**Interrupted interferon-alpha therapy**

In 1 patient, interferon therapy was stopped after 1 year, at which time the absolute lymphocyte count was 26.2% of the pretreatment count, and the serum M-CSF concentration was 200% of pretreatment levels. After stopping interferon therapy, the lymphocyte count gradually rose to 49.4% of the pretreatment count at 100 weeks. Resumption of interferon therapy at this point led to a fall in lymphocyte counts to 10.7% after a further 22 weeks on treatment. Both M-CSF and neopterin levels rose after commencing on interferon therapy, but fell to pretreatment levels when treatment was discontinued. Figure 5.2 shows the close correlation between serum M-CSF and neopterin levels, and the inverse relationship between these and the lymphocyte count, together with the timing of interferon therapy in this patient.

**Development of anti-interferon antibodies correlate with loss of clinical response and a fall in M-CSF levels**

Two patients developed neutralizing antibodies whilst on interferon therapy. Figure 5.3 shows that, in both patients, the rise in antibody titre is associated with a similar rise in the lymphocyte count (after an initial fall in response to starting interferon therapy), as well as a corresponding fall in M-CSF and neopterin levels. In these 2 patients, antibody titres showed a peak at about 20 weeks after starting therapy, and then began to fall, this reduction in antibody titre being accompanied by a gradual rise in M-CSF and neopterin levels, and an immediate reduction in the lymphocyte count.
Figure 5.2. Concomitant changes in (a) lymphocyte count, and (b) serum M-CSF and neopterin levels in one patient in whom interferon therapy was stopped for a year.
Figure 5.3. Concomitant changes (a) in lymphocyte count and circulating titres of anti-interferon antibodies, and (b) serum M-CSF and neopterin levels in patient CLL 12.
Figure 5.3. Concomitant changes in (c) lymphocyte count and circulating titres of anti-interferon antibodies, and (d) serum M-CSF and neopterin levels in patient CLL 5.
DISCUSSION

M-CSF is a homodimeric glycoprotein which enhances the survival, proliferation and differentiation of progenitor cells of the monocyte/macrophage lineage in vitro (Becker et al 1987), but which also has significant effects on mature effector cell function (Rettenmeir et al 1989). M-CSF acts on circulating monocytes and tissue macrophages to increase cell survival (Tushinski et al 1982), induce maturation linked antigens (HLA-DR, CD11b, CD11c and Fc receptors, Becker et al 1987), and enhance effector functions such as respiratory burst activity (Wing et al 1985), phagocytosis and microbial killing (Wang et al 1989), chemotaxis (Wang et al 1988) and tumour cell cytolysis, by both antibody-dependent and independent mechanisms (Sampson-Johhanes et al 1988, Nakoinz et al 1988, Munn et al 1990, Mufson et al 1989, Suzu et al 1989). The reported normal serum levels of M-CSF vary according to differences in the specific activities of the several forms of M-CSF employed (Yong et al 1992). In this study, circulating M-CSF levels in untreated patients with early stage CLL (456±205 U/mL) are not significantly different from that reported for normal controls by Hanamura et al, using the same ELISA (540±110 U/mL). In a previous report, 19 out of 21 patients with CLL were found to have elevated circulating M-CSF levels (Janowska-Wieczorek et al 1991). However, some of the patients in this study were at a more advanced stage of disease, and more than half were on treatment. In addition, these authors also reported that M-CSF levels were significantly lower in patients with only
increased lymphopoiesis, which would agree with the data presented here on patients with early stage CLL.

In these patients, interferon produces a significant increase in serum M-CSF levels, which, when followed serially in individual patients, is inversely correlated with the lymphocyte count. Loss of clinical response, either due to temporary interruption in interferon therapy, or to the development of anti-interferon antibodies, is associated with a concomitant fall in M-CSF concentrations to pretreatment levels. Serum neopterin levels also rise during interferon therapy; this has been previously reported by others (Lang et al 1984, Steiss 1991).

While activated macrophages are considered to be the sole cellular source of circulating neopterin (Huber et al 1984), it is not clear which cell type/s are responsible for the raised levels of M-CSF in patients receiving interferon. M-CSF is produced by fibroblasts, T cells, and bone marrow stromal cells in culture (Seelentag et al 1987, Takahashi et al 1988), and by monocytes and macrophages following exposure to cytokines such as gamma-interferon, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-alpha (Rambaldi et al 1987, Horiguchi et al 1987, Oster et al 1987). It is possible that both M-CSF and neopterin are synthesized by the same cells in response to interferon. On the other hand, interferon could act on other cell types, such as T cells, to induce M-CSF production (Takahashi et al 1988). This is unlikely because, although interferon is able to induce LAK activity, and to enhance NK activity in CLL, there is no
evidence for further T cell activation in patients receiving interferon (Chapter 4).

The observation that M-CSF and neopterin levels rise following initiation of interferon therapy suggests that mononuclear cell activation may be involved in immunological responses to interferon-alpha. How, and whether monocyte/macrophages exert direct, or indirect anti-tumour effects in patients receiving interferon-alpha, and the role of M-CSF in mediating these effects, remains unclear.

Monocytes/macrophages exposed to M-CSF in turn secrete cytokines including interleukin-1 (IL-1), granulocyte colony-stimulating factor (G-CSF), tumour necrosis factor (TNF) and interferon-alpha (Warren et al 1986, Motoyoshi et al, 1989). Hence M-CSF may play an important part in the cytokine feedback loops involved in the control, or potentiation, of immune responses.

There is evidence that elevated M-CSF levels in vivo lead to enhanced mononuclear cell function. Endogenous M-CSF levels are raised in conditions where there is activation of the reticuloendothelial system, such as immune thrombocytopenic purpura (Yong et al 1992). In nonhuman primates, administration of rhM-CSF induces a monocytosis, and enhances antitumour ADCC in circulating monocytes (Munn et al 1990). When administered to patients with lymphoma, hM-CSF enhanced monocyte functions such as migration into skin windows, priming of the respiratory burst, and phagocytosis/killing of candida (Khwaja et al 1991).

The results of Chapter 4 and 5 show that there is little evidence of T cell activation in response to interferon therapy
in early stage CLL, and that, although there is evidence for mononuclear cell activation the causal relationship between this, and the haematological responses seen, remains unclear. A reduction in the numbers of circulating leukaemic cells could also be due to changes in the homing behaviour and recirculation of CLL cells. Chapters 6 and 7 will address the adhesive properties and functions of CLL cells, in the context of stimulation with interferon-alpha and other cytokines.
CHAPTER 6

EXPRESSION OF ADHESION MOLECULES ON CLL CELLS AND
THE EFFECT OF CYTOKINE STIMULATION

INTRODUCTION

Leucocytes express a variety of adhesion promoting molecules on their cell surface which mediate the cellular interactions responsible for the maintainence of immune surveillance and the development of immune and non-immune inflammatory responses (Springer 1990). Cellular adhesion molecules are involved in antigen presentation, antigen dependent and independent lymphocyte activation, T-cell dependent B-cell activation, and cell-mediated cytotoxicity and conjugate formation (Springer et al. 1987, Dransfield et al. 1990). In the recruitment of leucocytes into inflammatory areas, adhesion receptors on circulating cells bind to their ligands on inflamed vascular endothelium, leading to cell adherence followed by migration into the extravascular tissues (Zimmerman et al. 1992). Leucocyte-endothelial interactions are also crucial to the regulation of lymphocyte homing and migration. Specific homing receptors on lymphocytes recognise tissue specific endothelial ligands on specialised endothelial cells lining the high endothelial venules in lymphoid tissue (Stoolman 1989, Berg et al. 1989).

Several families of adhesion molecules have been identified. The leucocyte integrin family (Larson & Springer 1990, Arnaout 1990) is composed of three heterodimeric membrane glycoproteins which share a common β subunit, β2, which is reactive with CD18 mAbs. The β2 subunit can associate with
three different α subunits, α1, α2 and α3, which are reactive with CD11a, CD11b, CD11c mAbs respectively, to form three different functional receptors. These adhesion molecules play a crucial role in the immune and inflammatory responses of leucocytes. CD11a/CD18, or lymphocyte function associated antigen-1 (LFA-1), is present on all leucocytes, and mediates a broad range of lymphocyte functions including homotypic aggregation, helper T-cell functions, T-cell mediated cytotoxicity, and adhesion to endothelium (Springer et al 1987). CD11a/CD18 also participates in several adhesive functions of neutrophils and monocytes such as ADCC and antigen presentation (Larson & Springer 1990). CD11b/CD18 or macrophage antigen-1 (Mac-1) and CD11c/CD18 or p150,95 mediate the adhesive functions of neutrophils and monocytes (Anderson et al 1986, Carlos & Harlan 1990).

Another integrin family are the 'very late activation antigens' (VLA, Hemler 1987). These are also αβ heterodimers which share a common β chain distinct from that used by the leucocyte integrins, and at least 6 unique α chains. Members of this family are found on both haemopoietic and non-haemopoietic tissues and function primarily as adhesion receptors for extracellular matrix components (Shimizu et al 1990), with the exception of VLA-4, which also binds to an inducible receptor on endothelial cells, termed VCAM-1 (Osborn et al 1989). The VLA receptors are involved in tissue organization, T-cell immune responses, while VLA-4 functions in lymphocyte adhesion to activated endothelium (Shimizu et al 1992), and may also be important in lymphocyte homing and recirculation (Holzmann et al 1989).
A recently recognised family of adhesion molecules is the selectin family, members of which have a common carbohydrate-binding lectin domain (Lasky 1992). These molecules play an important role in the interactions of leucocytes with endothelium. E-selectin, also known as endothelial leucocyte adhesion molecule-1 (ELAM-1) is expressed on stimulated endothelial cells (Bevilacqua et al. 1987), and P-selectin, or granule membrane protein-140 (GMP-140) is expressed on activated platelets (Johnston et al. 1989). L-selectin (leucocyte adhesion molecule-1, LAM-1) is expressed on all leucocytes and haemopoietic progenitor cells (Tedder et al. 1990, Kansas et al. 1985). L-selectin was initially characterized as the peripheral lymph node homing receptor in the mouse (recognized by the MEL-14 mAb, Gallatin et al. 1983), and is now also implicated in the extravasation of neutrophils into inflammatory sites (Lewinsohn et al. 1987, Jutila et al. 1989). Another class of cell adhesion molecules involved in lymphocyte homing is the Hermes group of antigens, reactive with CD44 mAbs, and which includes Pgp-1 and gp90\textsuperscript{Hermes} (Jalkanen et al. 1987, Picker et al. 1989, Berg et al. 1989). This is a group of cell surface glycoproteins which, in addition to mediating lymphocyte-HEV binding, may also be involved in cell-matrix interactions, and are found on many non-lymphoid cells including fibroblasts, glial cells and epithelial cells.

A third group of adhesion molecules comprises members of the immunoglobulin superfamily (Williams & Barclay 1988), which includes the molecules involved in antigen specific T cell activation, the antigen receptors of T cells, molecules of the major histocompatibility complex (MHC), CD4, CD8, CD2 and its
ligand, lymphocyte function associated antigen-3 (LFA-3). Other important members of this family include the ligands for LFA-1, intercellular adhesion molecule-1 (ICAM-1, Rothlein et al 1986a), ICAM-2 (Staunton et al 1989), and ICAM-3 (Fougerolles & Springer 1992), as well as the ligand for VLA-4, vascular cell adhesion molecule-1 (VCAM-1). ICAM-1 is found on a variety of haemopoietic and non-haemopoietic tissues including activated lymphocytes and vascular endothelium. ICAM-2 is restricted to vascular endothelium, as is VCAM-1, while ICAM-3 is expressed on leucocytes. ICAM-1 is important in many aspects of immune response generation, including antigen presentation and T cell mediated cytotoxicity, as well as in the adhesion and migration of leucocytes to, and across cytokine activated endothelium (Dougherty et al 1988, Makgoba et al 1988, Oppenheimer-Marks et al 1991).

The differential regulation of these adhesion molecules is crucial to the control of differentiation, activation and migration of leucocytes. For example, maturation of B and T cells is associated with the increase in expression of LFA-1 and L-selectin (Kansas & Dailey 1989). Conversely, phorbol ester stimulation of lymphocytes leads to rapid shedding of surface L-selectin in humans and MEL-14 in mice (Tedder et al 1990, Kishimoto et al 1990). In addition, mitogen stimulation of memory T cells and antigen-driven B cell activation leads to loss of L-selectin after several days (Tedder et al 1990, Kanof & James 1988). Memory T cells express a different adhesion phenotype from naive T cells, for example, higher levels LFA-1, CD2 and LFA-3, but lower levels of L-selectin (Sanders et al 1988). Many of the processes involved in lymphocyte maturation
and differentiation are under the control of a range of soluble growth and differentiation factors and these may be able to regulate the coordinate expression of these adhesion molecules.

Cell-cell adhesive mechanisms have also been implicated in the pathophysiology of leukaemias and lymphomas. Interactions between tumour cells and normal tissues, including vascular endothelium can influence tumour localisation and metastasis (Pals et al 1989). For example, the expression of L-selectin has been implicated in the dissemination of lymphomas (Sher et al 1988, Bargatze et al 1987). High expression of the Hermes antigen homing receptor was found to correlate with disseminated disease and advanced clinical stage in non-Hodgkin's lymphomas (Pals et al, 1989). Cell adhesion via LFA-1 may contribute to tissue invasion by lymphoma cells (Roos & Roosien 1987, Strauder et al 1989). Similarly, low expression of LFA-1 and ICAM-1 in CLL, in contrast to solid B-cell lymphomas might explain the differences in tumour localisation (Inghirami et al 1988, Boyd et al 1989). On the other hand, lack of expression of LFA-1, LFA-3 and ICAM-1, has also been suggested as a mechanism of tumour escape from immune surveillance mechanisms (Clayberger et al 1988, Gregory et al 1988).

In CLL, the malignant cells initially circulate in the bloodstream, with variable and progressive localization in lymphoid tissue. The expression of homing receptors and other adhesion molecules involved in lymphocyte interactions with endothelium may relate to patterns of disease dissemination. On the other hand, the resistance of CLL cells to cell-mediated cytotoxicity may be related to low or absent levels of surface adhesion structures necessary for effective conjugate formation with
effector cells. This chapter examines the surface expression of adhesion molecules on CLL cells, and the way in which this can be altered by exposure to interferon-alpha and other cytokines.
PATIENTS

19 previously untreated patients with CLL (Binet stages A-C) were studied. Control peripheral blood lymphocytes were obtained from laboratory personnel. 4 patients with Binet stage A disease were treated with interferon-alpha (3 MU subcutaneously 3 times per week).

In addition to these patients with CLL, 2 patients with untreated hairy cell leukaemia and 1 patient with prolymphocytic leukaemia were also studied. Samples from the patients with hairy cell leukaemia were kindly provided by Dr Matutes and Prof. D. Catovsky, Royal Marsden Hospital, London.

SPECIAL METHODS

Cell culture

E-rosette negative cells from patients and controls, isolated as described in Chapter 2, were suspended in RPMI/FCS at 5x10^5/mL and cultured in RPMI-1640 with penicillin, streptomycin and 10% FCS with or without addition of cytokines. After 18 hours cells were washed 3 times in RPMI/FCS and examined for surface antigen expression by indirect immunofluorescence as detailed in Chapter 2.
RESULTS

Expression of adhesion molecules on CLL cells

Freshly isolated E-rosette negative cells from patients were examined for surface phenotype by indirect immunofluorescence as detailed in Chapter 2. CLL cells from untreated patients expressed significant levels of L-selectin and CD44, but the expression of the integrins CD11a, CD11b, and CD11c was low or absent (Table 6.1). In all of 19 cases examined the leukaemic cells expressed L-selectin, but levels were variable, with 20.3±12.2% (mean±SD) of cells expressing the antigen (range 5-38%, median 16%). Levels of ICAM-1 expressed by CLL cells were highly variable; seven out of eleven patients examined had no detectable ICAM-1 on the cell surface, four others had levels of expression ranging from 10%- 37% (the median for all the patients was 5%). CD44 was also found on all 19 cases examined, and 82.9±13.6% of cells expressed the antigen. VLA-4 was also found on all of the five samples examined, with 56.5±10.3% of the cells expressing the antigen. Similarly, in five samples tested all five expressed HLA-DR (64.8±12.2% positive). In four samples tested only low levels of staining was seen for CD45RO, 18.1 ± 5.2% of cells were positive for the antigen.
<table>
<thead>
<tr>
<th></th>
<th>CD11a</th>
<th>CD11b</th>
<th>CD11c</th>
<th>L-Selectin</th>
<th>ICAM-1</th>
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<th>CD45RO</th>
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</tbody>
</table>

Table 6.1. Constitutive expression of adhesion molecules on CLL cells.

Freshly isolated, E-rosette negative cells from patients with B-CLL were examined for antigen expression using indirect immunofluorescence and flow cytometry.

aCases were considered positive for the antigen if >5% of cells were positive when gates were set such that the negative control stained with an isotype-matched antibody contained 5% positive cells.

bMean±SD
L-selectin expression on CLL cells is upregulated by cytokines in vitro

Following 18 hours incubation with cytokines, or medium as control, E-rosette negative cells from patients with CLL and normal controls were examined for L-selectin expression by indirect immunofluorescence using Leu-8 MoAb and RAM-FITC as detailed in Chapter 2, and analysed by flow cytometry. Figure 6.1 shows that interferon-alpha (500 u/mL) enhances the surface expression of L-selectin on E rosette negative cells from 2 representative patients with CLL, increasing both the mean antigen density of cells, and the proportion of cells expressing the antigen. The mean cell fluorescence (MCF) of IFN-incubated cells was increased to 330±127% (mean ±SD) of the expression on cells incubated with medium alone (n=13, p<.0005, Figure 6.2). In five patients, similar results were obtained using TQ-1 mAb to stain cells for L-selectin expression (data not shown). IL-4 (1ng/mL) and interferon-gamma (100U/mL) were also able to upregulate surface density of L-selectin on CLL cells after 24 hours incubation; the MCF of stimulated cells was increased to 218±119% (n=8, p<0.001) and 245±116% (n=5, p<0.001), respectively, of control levels (Figure 6.2). Figure 6.3 shows that the percentage of cells expressing the antigen is also increased, following incubation with each of the 3 cytokines.

Incubation of CLL cells with IL-1 (5U/mL), IL-2 (100µg/mL) or TNF-α (100U/mL) did not significantly alter L-selectin expression (Figure 6.3d).
Figure 6.1. Effect of interferon-alpha on L-selectin expression by CLL cells. E-rosette negative cells from 2 patients were labelled by indirect immunofluorescence using Leu-8 mAb and RAM-FITC, following 18 hours incubation with interferon-alpha (500 U/mL) (panels c,f) or medium as control (panels b,e). Negative controls (panels a,d) were stained with an isotype-matched irrelevant antibody. Similar results were obtained for 11 other patients.
Figure 6.2. Induction of L-selectin expression on CLL cells by cytokines. E-rosette negative cells from 13 patients with CLL were examined for L-selectin expression by indirect immunofluorescence, and mean cell fluorescence (MCF) was determined by flow cytometry. The data shows the effect of interferon-alpha (500 U/mL), interferon-gamma (100 U/mL) and IL-4 (1 ng/mL) on the MCF of cells (mean±SD, expressed as a percentage of the expression on control samples incubated with medium alone).
Figure 6.3. Effect of cytokines on the percentage of cells expressing L-selectin. The percentage of cells positive for L-selectin was obtained by setting gates on the flow cytometer such that 5% of cells were positive in the negative control samples. Data is given for individual patients, and shows the effect of (a) interferon-alpha on the expression of L-selectin by CLL cells in 13 patients.
Figure 6.3. Effect of (b) IL-4 (8 patients), and (c) interferon-gamma (5 patients) on the percentage of CLL cells expressing L-selectin.
Figure 6.3 (d). Effect of IL-1 (5U/mL), IL-2 (100µg/mL) and TNF (100U/mL) on L-selectin levels of CLL cells from 1 representative patient.
The effect of interferon-alpha and other cytokines on L-selectin expression was also examined in peripheral blood mononuclear cells from normal donors. In contrast to CLL cells, there was no significant effect of interferon-alpha or other cytokines on surface L-selectin expression in normal cells (Table 6.2). In addition, hairy cells from 2 patients with hairy cell leukaemia (Table 6.3) and the cells from 1 patient with prolymphocytic leukaemia did not demonstrate upregulation of L-selectin following incubation with interferon-alpha.

CLL cells were also examined for surface expression of other adhesion receptors, before, and after exposure to various cytokines. Levels of CD11a, CD11b, CD11c, CD44 or ICAM-1, were not altered after 18 hour exposure to IL-1, IL-2, IL-4, interferon-alpha or interferon-gamma (Table 6.4).
Table 6.2. Effect of cytokine stimulation on L-selectin expression of normal peripheral blood B and T cells.

Data, expressed as the percentage of cells expressing the antigen, are given for individual experiments.
Table 6.3. Effect of cytokines on surface expression of L-selectin in hairy cells from 2 patients with hairy cell leukaemia. E-rosette negative cells were treated with cytokines or medium as control as detailed in the text, and examined for L-selectin expression by indirect immunofluorescence, using Leu-8 mAb.

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MCF % positive</td>
<td>MCF % positive</td>
</tr>
<tr>
<td>control</td>
<td>7.2 50.0</td>
<td>23.5 25.0</td>
</tr>
<tr>
<td>IFN-α</td>
<td>9.0 56.0</td>
<td>22.5 27.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>6.3 44.7</td>
<td>ND ND</td>
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Table 6.4. Effect of cytokines on surface expression of adhesion molecules in CLL cells.

E-rosette negative cells from patients with CLL were examined for expression of adhesive receptors following 18 hours incubation with cytokine or medium as control. Data, expressed as the percentage of cells staining positive for the antigen, is given for 2 separate experiments.

<table>
<thead>
<tr>
<th></th>
<th>LFA-1 Expt.1</th>
<th>LFA-1 Expt. 2</th>
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<th>ICAM-1 Expt. 2</th>
<th>CD44 Expt. 1</th>
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**L-selectin expression is downregulated following exposure to phorbol esters and other mitogens**

Following 18 hours incubation with TPA (100ng/mL), CLL cells demonstrated a significant decrease in the cell surface expression of L-selectin (-94.2±31.2% change in MCF, n=3, p<0.001). Similar results were obtained when calcium ionophore, poke-weed mitogen or anti-IgM antibodies were used (Figure 6.4).

**Dose dependence and kinetics of L-selectin induction by interferon-alpha, IL-4 and interferon-gamma**

E-rosette negative cells from 3 patients with CLL were incubated with increasing concentrations of interferon-alpha for 18 hours before L-selectin surface antigen density was measured. Figure 6.5a shows that the induction of surface L-selectin levels on CLL cells by interferon-alpha occurred in a dose dependent manner. Significant induction of the antigen was seen at 0.5 U/mL, with near maximal effects occurring at 500 U/mL. Similar dose dependent effects of IL-4 and interferon-gamma were also seen (Figure 6.5b,c).

In order to determine the kinetics of L-selectin induction, E-rosette negative cells from 2 patients with Stage A CLL were incubated with interferon-alpha (500 U/mL) and IL-4 (10 ng/mL) for varying periods of time. In the presence of either interferon-alpha or IL-4, an increase in surface L-selectin expression was evident after 4 hours, and antigen expression continued to rise for up to 48 hours (Figure 6.6). A small increase in cell surface L-selectin was also seen in cells incubated with medium alone, but this did not reach significance.
Figure 6.4. Downregulation of L-selectin expression on CLL cells following exposure to phorbol esters and mitogens.

CLL cells were incubated with TPA (100ng/mL), calcium ionophore (Cal, 10µg/mL), pokeweed mitogen (PWM, 10µg/mL), anti-IgM (5µg/mL) or medium as control for 18 hours, before being examined for L-selectin expression by indirect immunofluorescence. Data, expressed as the percentage of cells positive for the antigen, are the mean of duplicate samples in 1 representative experiment.
Figure 6.5. Dose dependent induction of L-selectin on CLL cells by cytokines. Mean channel fluorescence (MCF, expressed as percentage of levels on control cells incubated with medium alone) of CLL cells was determined after 18 hours incubation with (a) interferon-alpha (500 U/mL). Data are mean±SD of 3 patients.
Figure 6.5. Dose dependent induction of L-selectin on CLL cells by (b) IL-4 (1 ng/mL, 2 separate patients) and (c) interferon-gamma (100 U/mL, mean±SD of 3 patients)
Figure 6.6. Time course of the induction of L-selectin on CLL cells by cytokines. CLL cells were incubated with interferon-alpha (500 U/mL), IL-4 (1 ng/mL) or medium as control, and analysed for L-selectin expression at 4, 24 and 48 hours. Data, expressed as MCF, are the mean of duplicates, and are given for 2 individual patients (a) and (b).
**Induction of L-selectin on CLL cells is protein synthesis dependent**

E-rosette negative cells from 3 patients with CLL were incubated with interferon-alpha (500 U/mL), in the presence or absence of cycloheximide (25mM) for 18 hours. Figure 6.7 shows that cycloheximide abolished the induction of L-selectin on CLL cells by both interferon-alpha and IL-4, suggesting that the upregulation of L-selectin surface antigen by these cytokines requires protein synthesis. Interestingly, in the presence of cycloheximide, surface expression of L-selectin was depressed to levels below those found on control cells incubated with medium alone, suggesting that receptors are being turned over continually, and that protein synthesis is required to maintain basal levels of expression.

**Lack of induction of L-selectin expression by interferon-alpha in vivo**

In order to determine if L-selectin levels on leukaemic cells could be modulated by interferon-alpha *in vivo*, surface L-selectin expression was also monitored in 4 patients with Binet stage A CLL undergoing interferon therapy. The MCF of peripheral blood CLL cells demonstrated a small but insignificant decrease following interferon therapy (to 65 ± 36% of initial levels, NS by paired t-test), while the actual percentage of cells expressing the antigen showed no change (Figure 6.8).
Figure 6.7. The induction of L-selectin expression on CLL cells is protein synthesis dependent. CLL cells were analysed for L-selectin expression as detailed above after 18 hours incubation with interferon-alpha (500 U/mL) or IL-4 (1 ng/mL), in the presence or absence of cycloheximide (CHX) 25 mM. Data is expressed as percentage change in MCF, as compared with control samples incubated with medium alone (mean±SD, n=3).
Figure 6.8. Effect of interferon-alpha therapy on L-selectin expression \textit{in vivo} in 2 patients with Stage A CLL. E-rosette negative peripheral blood mononuclear cells were examined for L-selectin expression prior to, and at various times after starting on interferon therapy. Data are given as the percentage of cells expressing the antigen.
DISCUSSION

The constitutive pattern of adhesion molecule expression on CLL cells, that is, significant but variable levels of L-selectin, and high levels of CD44 in contrast to low levels of CD11a, CD11b and CD11c has implications for the homing behaviour and degree of immune competence of these malignant cells. Both L-selectin and CD44 are involved in lymphocyte homing, and are upregulated during cell maturation. L-selectin is the peripheral lymph node homing receptor mediating tissue specific binding of lymphocytes to high endothelial venules in peripheral lymphoid tissue (Gallatin et al 1983, Tedder et al 1990), while CD44 recognizes a class of lymphocyte surface receptors involved in lymphocyte-HEV interactions, which may or may not be tissue specific (Berg et al 1989). Significant surface expression of these receptors suggests that CLL cells may be competent to interact with HEV, and could explain the patterns of dissemination in advanced disease (Pals et al 1989).

The low levels of CD11a (LFA-1) expression compared with the high levels on normal peripheral blood lymphocytes (Kurzinger et al 1981) has been noted by previous workers (Clayberger et al 1987, Inghirami et al 1988), and has been suggested to be one possible mechanism whereby the malignant cells escape immune surveillance mechanisms. Similarly, low expression of CD11a (LFA-1) could also account for abnormalities in B cell function in this disease, as CD11a, quite apart from functioning as an adhesion molecule, has been shown to be important in signalling and cell activation (Wacholtz et al 1990). The low levels of ICAM-1 expression on CLL cells described here agree with a
previous report by Stauder et al (1989) who also found that lack of ICAM-1 expression in B-cell lymphoproliferative disorders was correlated with spread of malignant cells into the bloodstream. ICAM-1 is either absent, or expressed at very low levels on normal circulating B cells, but is induced to high levels following mitogenic stimulation (Wawryk et al 1989). ICAM-1 is also involved in homotypic and heterotypic lymphocyte adhesion (Schulz et al 1988), and the low levels found on CLL cells may, again, relate to escape from immune anti-tumour mechanisms.

An important finding in this chapter is that interferon-alpha was able to increase the cell surface expression of L-selectin on CLL cells from all patients studied. Similar effects were also seen when CLL cells were exposed to Interferon-gamma or IL4 in vitro. Induction of LAM-1 expression occurred rapidly in a dose-dependent manner and required protein synthesis. The functional significance, and pathophysiological implications of the induction of L-selectin expression on CLL cells by interferon-alpha, and other cytokines are not clear. The expression of this antigen on normal cells is regulated in a complex manner by activation and differentiation signals. Mitogen stimulation of MNCs and memory T cells leads to loss of surface L-selectin after several days (Tedder et al 1990, Kanof et al 1988). Similarly, antigen-driven B cell maturation/differentiation leads to loss of L-selectin, and L-selectin negative peripheral blood B cells from normal individuals express an activated phenotype, and have the ability to respond to pokeweed mitogen (Kansas et al 1985). Thus L-selectin may be specifically downregulated during cell division or long term activation. In addition to this differentiation related loss of surface L-selectin which occurs over days, phorbol
ester stimulation of lymphocytes leads to rapid shedding of surface L-selectin in humans and MEL-14 in mice (Tedder et al 1990). Conversely, partial activation of lymphocytes increases the expression of L-selectin, while strong activation leads to blast morphology, with concomitant loss of L-selectin expression. Hence stimuli which may not suffice to drive cells to proliferate may nevertheless change their homing behaviour. The increase in L-selectin expression on CLL cells in response to interferon-alpha seen in vitro here may reflect an alteration in the state of activation of these cells.

Cell activation leads to alterations in surface antigen expression, with consequent changes in the recirculation patterns and homing behaviour of lymphocytes (Willerford et al 1989, Pals et al 1989). Enhanced expression of the L-selectin receptor on CLL cells following cytokine stimulation may lead to alterations in the homing behaviour of these cells. The question arises as to whether the L-selectin receptor on CLL cells is functionally normal, and competent to mediate lymphocyte binding. The L-selectin receptor on CLL cells is down-modulated in a similar way to that on normal lymphocytes following cell activation by mitogens, as demonstrated in this chapter, and in other work (Spertini et al 1991). In addition, the L-selectin receptor expressed on CLL cells has been shown to resemble the Mr 74,000 isoform of the glycoprotein expressed on normal lymphocytes (Spertini et al 1991). These workers also showed that the binding of CLL cells to HEV in the frozen section assay of Stamper and Woodruff (1976) was correlated with L-selectin expression, and was blocked by a mAb to L-selectin, LAM 1-3. These observations suggest that the L-selectin antigen on CLL cells is functionally
normal, and that increased surface expression would lead to enhanced binding to peripheral lymphoid HEV, resulting in more cells leaving the circulation. This would account for the reduction in malignant cells in the peripheral circulation seen in some CLL patients treated with interferon-alpha. The lack of effect of interferon-alpha on L-selectin expression in vivo cannot be due to suboptimal plasma concentrations of interferon-alpha because the dose range which is effective in vitro (greater than or equal to 0.5U/ml) correlates well with the concentrations estimated in vivo (ABPI Data Sheet Compendium. 1989; Datapharm publications p. 1739.) in patients on treatment. It is more likely that the lack of upregulation of surface L-selectin on peripheral cells following interferon-alpha therapy in vivo reflects the fact that those cells that have increased cell surface L-selectin expression have left the circulation.

Evidence that interferons may directly influence lymphocyte recirculation comes from studies in mice in which systemic administration of interferon led to lymph node enlargement (Schattner et al 1985). In another report, rat lymphocytes treated with interferon-alpha, either ex vivo or in vivo, exhibited altered recirculation patterns, with increased retention in lymph nodes (Kimber et al 1987, Hein et al 1988). Another group has reported that murine lymphocytes exposed to interferon-gamma in vivo showed increased binding to HEV in vitro, but levels of MEL-14 on the in vivo treated lymphocytes were unchanged (Hendricks et al 1989).

Finally, apart from its role in lymphocyte homing, L-selectin may also be important in lymphocyte differentiation and maturation. L-selectin is upregulated on B cells during
development (Kansas et al 1989) and anti-L-selectin antibodies can inhibit B-cell differentiation but not proliferation (Murakawa et al 1991). Interferon-alpha has also been reported to affect the growth and differentiation of CLL cells (Ostlund et al 1986). Hence the induction of L-selectin on CLL cells may be related to cytokine mediated changes in the functional and/or maturational state of the cells. The effect of interferon-alpha on the activation and maturational status of CLL cells is addressed in Chapters 8 and 9, which deal with cell proliferation and cell death, in the context of cytokine stimulation. The following chapter deals with the question of cell adhesion and homing, and examines the way in which CLL cells interact with cultured human endothelium in vitro, and the effects of interferon-alpha on cell adhesion.

Although interferon-alpha treatment appears to increase L-Selectin expression on the CLL cells, this effect may be mediated by secondary cytokine release by contaminating monocytes. The presence of significant numbers of contaminating monocytes in these cultures, and the fact that interferon-alpha activates monocytes in vivo (See Chapter 5) are consistent with this hypothesis.
CHAPTER 7
INTERACTION OF CLL CELLS WITH CULTURED HUMAN HIGH ENDOTHELIUM AND UMBILICAL VEIN ENDOTHELIUM IN VITRO: EFFECT OF CYTOKINES AND THE ROLE OF ADHESION MOLECULES

INTRODUCTION

Lymphocytes recirculate continuously between the blood and peripheral lymphoid tissues via a network of lymphatic vessels which empty into the thoracic duct (Gowans & Knight 1964). This recirculation is essential to the maintenance of immune surveillance, as well as to the generation of appropriately directed immunological memory. Lymphocytes extravasate from the blood by binding to endothelium at specialized post-capillary sites termed high endothelial venules (HEV). Lymphocyte recirculation and localisation is thus dependent upon the differential regulation of lymphocyte adhesion (Picker & Butcher 1992).

Similarly, adhesive interactions between tumour cells and normal tissues can influence tumour localisation and dissemination (Weiss et al 1988, Albelda et al 1990, Hart & Saini 1992). The pattern of dissemination of lymphoid malignancies resembles the homing behaviour of the normal counterpart cell type (Pals et al 1989, Bargatze et al 1987, Sher et al 1988), for example, gastrointestinal or cutaneous lymphomas tend to spread to, or recur in a tissue specific manner, ie to mucosal sites or skin respectively. B cell chronic lymphocytic leukaemia (CLL) is characterized by accumulation of a clonal proliferation of small
lymphocytes in the peripheral blood (Catovsky & Foa 1990). In early stages of the disease, these cells are found mainly in the peripheral circulation, but the disease progresses to involve lymphoid and nonlymphoid tissue. The specific localisation of these cells in peripheral lymphoid tissue and in the bone marrow suggests that similar adhesive mechanisms which regulate the traffic and homing behaviour of normal lymphocytes may also operate to influence the spread of these tumour cells.

Lymphocyte interactions with vascular endothelium, both in the regulation of organ-specific homing (Gowans & Knight 1964), and in the context of inflammatory responses, are mediated by cell surface molecules (Shimizu et al. 1992). The appropriate trafficking of lymphocyte populations to selective regional lymphoid organs is based on recognition events between lymphocytes and high endothelial venular cells (HEVC) (Gallatin et al. 1983, Stamper & Woodruff 1976). Lymphocyte surface receptors which are known to participate in this interaction include L-selectin, VLA-4/LPAM-2, CD44 and LFA-1 (Stoolman 1989, Duijvestijn & Hamann 1989). L-selectin, also termed leucocyte adhesion molecule-1 (LAM-1) or lectin adhesion molecule-1 (LECAM-1), originally identified in the mouse by the MEL-14 monoclonal antibody (Gallatin et al. 1983) is the best characterized homing receptor and mediates lymphocyte binding to peripheral lymph node HEV (Bowen et al. 1989, Geoffrey & Rosen 1989, Watson et al. 1990). An endothelial ligand for L-selectin has recently been purified and cloned from murine lymph node (Lasky et al. 1992). LPAM-2, which is the murine homologue of VLA-4, is thought to participate in lymphocyte homing to mucosal lymphoid organs, such as Peyer's Patches (Holtzmann et
Acting in concert with these homing receptors, are accessory molecules, such as lymphocyte function associated antigen-1 (LFA-1) which augment lymphocyte adhesion to high endothelium without imparting organ selectivity (Pals et al 1988).

Lymphocyte interactions with endothelium are not only the result of tissue-specific receptor-ligand binding, but can also be dramatically altered by inflammation. Inflammatory cytokines, such as tumour necrosis factor (TNF) and interleukin-1 (IL-1), as well as T cell derived lymphokines such as interleukin-4 (IL-4) and gamma-interferon (Interferon-gamma) activate endothelial cells, resulting in either increased expression of adhesive ligands for lymphocyte binding, such as intercellular adhesion molecule-1, (ICAM-1)(Pober et al 1986) or de novo induction of molecules such as endothelial leucocyte adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule-1 (VCAM-1)(Bevilacqua et al 1987, Osborn et al 1989). Such ligand induction by cytokines, which occurs *in vitro*, and can be observed in a variety of natural and experimental lesions *in vivo* (Mantovani & Dejana 1989, Pober & Cotran 1990), leads to enhanced binding of lymphocytes to cultured endothelium *in vitro* (Cavender et al 1986, Yu et al 1985) and increased lymphocyte extravasation *in vivo* (Duijvestijn et al 1988). Adhesion molecules thought to mediate tissue-specific homing under normal conditions, such as L-selectin and VLA-4, also contribute to lymphocyte binding to inflamed endothelium, together with other more general adhesion molecules such as LFA-1 (Shimizu et al 1992, Carlos & Harlan, 1990).

*In vitro* studies of lymphocyte adhesion to high endothelium have hitherto largely been conducted using the frozen section
assay (Stamper & Woodruff, 1976), in which lymphocytes adhere to the cut surface of HEV in cryostat sections of rat lymph nodes (Gallatin et al. 1983). Such studies have provided the *in vitro* basis for the current theories of lymphocyte homing (Stevens *et al.* 1982), and have led to the identification and characterization of organ-specific homing receptors and the corresponding tissue-specific ligands on HEV, termed vascular addressins (Butcher *et al.* 1980, Streeter *et al.* 1988). One disadvantage of the frozen section assay is that it uses nonviable endothelium, and hence is limited in yielded information about the dynamic interactions between lymphocytes and HEV, particularly in the context of cytokine stimulation. Cultured human umbilical vein-derived endothelial cells (HUVEC) are widely used in studies of leucocyte adheison (Haskard *et al.* 1986). These relatively undifferentiated cells, however, differ from HEVC in morphology, phenotype and function (Kraal *et al.* 1986). Recently, there have been successful attempts to isolate and culture HEVC from rat lymph nodes (Ager 1987). This chapter describes the development of a novel system for the isolation and culture of human high endothelial cells from tonsils, using a modification of the method of Ager (1987). These tonsillar derived high endothelial cultures have been used to study and characterize the binding of CLL cells to human HEVC, comparing this with adhesion to the relatively non-specialised HUVEC.
SPECIAL METHODS

Human umbilical vein endothelial cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by collagenase treatment (0.1% in PBS, Jaffe et al, 1976), and grown to confluence in fibronectin (2 mg/cm²)-coated Falcon tissue culture flasks in IMDM with 20% heat inactivated FCS, ECGS (25 mg/mL) and heparin (25 U/mL). HUVEC cultures were identified by cobblestone morphology, and characteristic granular staining with von Willebrand Factor (vWF) antigen (Jaffe et al, 1976). The cells were passaged using 0.05%trypsin/0.01%EDTA in PBS, and seeded in fibronectin coated 96-well tissue culture plates (Falcon) at 2x10³ cells/well and grown to confluence (5-7 days) for adhesion experiments. Cells at passages 1-5 were used.

Human tonsillar high endothelial venular cell (HEVC) isolation and culture

Human tonsils were obtained within 1 hour of surgical removal during routine tonsillectomies. After washing once with RPMI, tissue was chopped finely, resuspended in RPMI and allowed to settle for 2-3 minutes. The lymphocyte-rich supernatant was removed and used as a source of tonsillar lymphocytes (see below). The chopped tissue was resuspended in RPMI and the procedure repeated until the supernatant was clear. The tissue was then incubated with 0.2% collagenase for 20 minutes at 37°C on a rotary mixer. At this stage, tissue was put through wire gauze, and plated in RPMI with 10% heat-inactivated FCS,
antibiotics and ECGS (100 ng/mL) onto fibronectin-coated tissue culture petri dishes (Falcon). After 24 hours, nonadherent cells were carefully aspirated, and replaced with fresh medium. This was repeated at 48 hours. Endothelial cells could be distinguished as single, polygonal cells which adopted a bipolar morphology, becoming aligned in parallel, as cultures approached confluence. Cultures reached confluence at 10-14 days, and were passaged using 0.05% trypsin /0.01% EDTA in PBS, and plated at 25-50% confluent density into 96-well fibronectin coated tissue culture plates (Falcon). Cultures reached confluence in approximately 5 days, and were used within 48 hours for adhesion assays.

**Lymphocyte isolation and labelling**

Peripheral blood mononuclear cells (PBM) were purified from heparinised venous blood by Ficoll centrifugation over Lymphoprep, washed in RPMI/10% FCS, and pelleted by centrifugation. Tonsillar lymphocytes were isolated by harvesting the lymphocyte-rich supernatant from the high endothelial cell preparation as detailed above, and layering onto Ficoll gradients as described above for PBM. In order to label cells for adhesion assays, $^{51}$Cr (1 mCi/mL, 1 μCi/10$^6$ cells) was added to the cell pellet (approximately 30-60X10$^6$ cells). After incubation at 37°C for 1 hour, the cells were washed 4 times in large volumes of RPMI/10% FCS, and resuspended at 10$^6$/mL in RPMI/10% FCS for adhesion assays.
**Neutrophil isolation and labelling**

Neutrophils were purified from venous blood taken into EDTA (2 mM) by double density centrifugation (Histopaque 1119 and 1077, Sigma Diagnostics), and washed twice in PBS with 5 mM glucose (PBS/glucose). Neutrophils obtained by this method were >95% pure and >99% viable by trypan blue exclusion. Purified neutrophils, at 15-20X10^6/mL in PBS/glucose, were incubated with 51Cr (2 μCi/10^5 cells) at 37°C for 60 minutes, with gentle agitation. Following 3 washes with large volumes of PBS/glucose, neutrophils were resuspended at 10^6/ml in RPMI/5% FCS for adhesion assays.

**Uptake of 35S by endothelial cells**

Confluent monolayers of HUVEC or HEVC in 96 well plates were incubated overnight at 37°C with 5 μCi Na_2^35SO_4/well. Monolayers were then washed with warm medium to remove unincorporated ^35S, and cell-associated radioactivity determined by scintillation counting after solubilizing cells with detergent (1% NP40, Sigma Chemicals).

**Phenotyping of endothelial cells**

Immunofluorescent staining of cytospin preparations

Immunofluorescent labelling of vWF antigen was performed on cytospin preparations of first passage cells, or on cells grown in culture chambers mounted on glass slides (Labtek Tissue Culture Chamber/Slide, ICN Biomedicals Ltd., High Wycombe, Bucks, UK). Cells were fixed in 70% methanol at 4°C for 20 minutes, and stained immediately. Slides were incubated with anti-vWF monoclonal antibody (1:200, Dakopatts) overnight at 37°C, and
washed 3 times with PBS, before incubation with FITC-RAM (1:100) for a further 30 minutes at 37°C. After a further 4 washes with PBS, nuclei were counterstained with propidium iodide (room temperature for 10 seconds), then washed again. Slides were mounted in PBS/glycerol, and viewed under a fluorescent microscope.

**Staining of endothelial cells in situ by enzyme immunoassay**

An enzyme immunoassay, modified after the method used by Smith *et al* (1988) was used to quantitate surface adhesion molecule expression on endothelial cells in culture. Confluent endothelial monolayers in 96 well plates were fixed using 1% paraformaldehyde in PBS for 15 minutes at room temperature. The wells were washed 3 times with PBS and incubated in 2% bovine serum albumin (BSA) for 30 minutes. After removal of BSA, mAb was added and the plates were incubated at 37°C for 1 hour, washed 3 times with PBS, and incubated for 1 further hour at 37°C with peroxidase-conjugated goat anti-mouse Ig. After another 3 washes, substrate was added (0.8 mg/mL of OPD in citrate phosphate buffer, pH 5, with 0.03% H2O2). The reaction was stopped with 2M H2SO4, and the optical density of the wells was read at 405 nm on a microplate ELISA reader (Dynatech Laboratories).

**Assays of leucocyte adhesion to endothelial monolayers**

Confluent HUVECs in 96-well microtitre plates were washed once with warm medium before being used in adhesion assays. 51Cr labelled cells (1 X 10^5 cells in 100 mL/well) were added to the wells. At least 6 wells were used for each data point. At the end
of the incubation period (30 minutes for neutrophil adhesion, 60 minutes for lymphocyte adhesion at 37°C), nonadhered cells were removed by 3 gentle washes with warm medium, and all washes were collected for counting in a gamma counter. At the end of the washing procedure, wells were examined using an inverted microscope to check that there was no disruption of the endothelial monolayers. Adherent cells and endothelium were solubilized with detergent, and the lysate, together with 2 washings, counted in a gamma counter. In some experiments, confluent endothelial cultures were preincubated with IL-1β (10 U/mL) or medium as control for 4 hours, and washed prior to being used for adhesion assays. In experiments examining the role of CD18 in lymphocyte adhesion, a saturating concentration (10 mg/mL) of mAb MHM23 (anti-CD18), or an isotype-matched control antibody, was added to lymphocyte suspensions for 5 minutes at room temperature prior to the adhesion assay, and antibodies were present throughout the incubation. For each experiment, spontaneous release of ⁵¹Cr by the labelled cells was determined by incubating aliquots of cell suspensions for the same length of time, following which the cells were centrifuged down and both the supernatant and the pelleted cells were removed and counted. In all experiments, spontaneous release was <5%, that is, >95% of counts were cell associated. The percentage of adherent cells was calculated from the formula:

\[
\text{% lymphocytes bound} = \frac{\text{radioactivity (cpm) in lysate}}{\text{total radioactivity (cpm) added to well}} \times 100
\]
The total radioactivity (cpm) added to each well, obtained by adding the radioactivity (cpm) of the nonadherent fraction to that of the cell lysate, was always within 5% of the radioactivity (cpm) measured in aliquots of the original suspension which was added to the wells.

Frozen section assay of lymphocyte adhesion to HEV
Cryostat sections of human lymph node were kindly supplied by Linda Wilkinson, Department of Rheumatology Research, UCL Medical School. Cryostat sections of monkey lymph nodes (BioDiagnostics, UK) were used in this assay, which was carried out according to the method of Butcher et al (1980). Briefly, lymphocytes were preincubated with interferon-alpha or medium as control for 18 hours, washed and resuspended at $7 \times 10^6$/mL in RPMI with 1% BSA, then placed on unfixed sections of monkey lymph nodes in wax pencil circles 1.6 cm in diameter ($7 \times 10^5$ cells in each circle), and incubated for 30 minutes with gentle agitation (60 rpm on a rotator). The medium with nonadherent cells was then decanted off, and the slides fixed in cold PBS with 1% glutaraldehyde. After fixation, nonadherent cells were gently rinsed off with PBS. All steps were carried out at 7°C in a cold room. Immediately adjacent sections were used for test (interferon-alpha treated) and control cells, and at least triplicate slides were used for each condition. Lymphocyte adherence to HEV was quantitated by counting the number of lymphocytes adhering to the same venule in each section under light microscopy, and expressing this as the number of lymphocytes per small square within the grid of an eyepiece graticule.
RESULTS

Characterization of human HEVC
Cultured human tonsillar HEVC were identified by the selective uptake of radioactive sulphur, and by phenotypic analysis.

Radioactive sulphur uptake
Confluent monolayers of HEVC or HUVEC were incubated with $^{35}$S (5 µCi/well) overnight (18 hours), and the amount of $^{35}$S incorporated was determined as outlined above. In order to standardise for cell numbers, cells from representative wells were removed by trypsinisation, and counted using a Neubauer cell counting chamber. Confluent cultures contained equivalent numbers of cells for both HEVC and HUVEC (approximately 5 $\times$ 10^4/well). As shown in Table 7.1, HEVC cultures exhibited levels of $^{35}$S uptake which were at least 5 times higher than those seen with HUVEC.

Expression of cell surface adhesion molecules
Surface antigen expression was quantitated on confluent endothelial monolayers in 96 well plates using an enzyme-linked immunosorbent assay, and measured in arbitrary absorbance units. The basal expression of all 3 endothelial adhesive ligands ICAM-1, ELAM-1 and VCAM-1 was significantly higher on cultured HEVC, when compared with HUVEC (Table 7.2). ICAM-1 expression on HEVC was 357±49% of that on HUVEC (p<.005), while ELAM-1 and VCAM-1 levels were 473±51% (p<.0005) and 257±46% (p<.025), respectively, of the levels found on HUVEC (Table 7.2).
The effect of cytokine stimulation on the surface levels of these adhesion receptors was examined by incubating confluent monolayers with IL-1β (10 U/mL) for 4 hours prior to carrying out the immunoassay for surface antigens. HEVC and HUVEC demonstrated marked differences in their antigenic response to cytokine stimulation. IL-1 stimulation of HUVEC increased ICAM-1 expression to 654±200% of basal levels, and ELAM-1 expression to 768±213% of resting levels (p<.0005, n=4 for both), while VCAM-1 levels demonstrated a more modest increase to 174±5% of basal levels (N.S. by paired t-test, Table 7.2). In contrast, HEVC failed to demonstrate any significant change in the expression of ICAM-1, ELAM-1 or VCAM-1, following exposure to IL-1. Interferon-alpha (10-1000 U/mL for up to 24 hours) had no effect on HUVEC or HEVC expression of these adhesion molecules (data not shown). In order to control for nonspecific changes in the binding of surface membrane receptors, the surface expression of vWF was measured as a control; Table 7.2 confirms that this did not change on either HUVEC or HEVC, following exposure to cytokines.

Expression of vWF antigen
HUVEC and HEVC were cultured on glass slides (Lab-tek tissue culture slides) to semi-confluence and fixed for 20 minutes using 70% methanol at 4°C, in order to permeabilized the cells. Staining for vWF antigen was carried out using indirect immunofluorescence. Preparations of HEVC revealed no staining for vWF Ag, in contrast to HUVEC, which showed strong granular positivity.
Table 7.1. Uptake of $^{35}$S by HUVEC and HEVC

Confluent cultures in 96-well plates were incubated overnight with 5 $\mu$Ci of $^{35}$S per well, washed to remove unincorporated $^{35}$S, and subsequently harvested by detergent lysis. Incorporated radioactivity was measured, and expressed as cpm per $10^4$ cells.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>cpm bound / $10^4$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HUVEC</td>
</tr>
<tr>
<td>1</td>
<td>438</td>
</tr>
<tr>
<td>2</td>
<td>223</td>
</tr>
<tr>
<td>3</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>ICAM-1</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>HUVEC - basal *</td>
<td>61±13</td>
</tr>
<tr>
<td>- IL-1 *</td>
<td>397±14</td>
</tr>
<tr>
<td>HEC - basal **</td>
<td>252, 184</td>
</tr>
<tr>
<td>- IL-1 **</td>
<td>273, 201</td>
</tr>
</tbody>
</table>

Table 7.2. Surface expression of endothelial adhesion molecules on cultured human HUVEC and HEVC.

Data is in arbitrary absorbance units, and is the mean±SE of 4 experiments*, or is given individually**, for 2 out of the 4 experiments in which the parameter was measured.
**Leucocyte adhesion to cultured HEVC**

1. **Lymphocytes.**

Under unstimulated conditions, the adherence of PBL to cultured HUVEC was 18.5±0.9% (mean±SD, n=8). Percentage adhesion to cultured HEVC was consistently higher in all experiments (33.0±6.8% when compared with adhesion to HUVEC, p<.01, n=8, Figure 7.1). In 2 experiments, tonsillar lymphocytes (TLs) were also tested for their ability to bind to HEVC and HUVEC. In both experiments, TLs demonstrated higher levels of binding to HEVC (24.1% adhesion to HEVC as compared with 18.2% to HUVEC in experiment 1, and 18.2% adhesion to HEVC as compared with 15.4% adhesion to HUVEC in experiment 2). Hence, under unstimulated conditions, lymphocytes demonstrate selective binding to cultured HEVC.

2. **CLL cells.**

E-rosette negative cells were obtained from untreated patients at various stages of disease. CLL cells also demonstrated higher levels of binding to HEVC when compared with HUVEC, although this difference was not as marked as that seen with PBL, and did not reach significance in this series of experiments. The adhesion of CLL cells to HUVEC under unstimulated conditions was 16.3±2.9% (mean±SEM, n=21) while that to HEVC was 25.7±7.3% (n=9) (Figure 7.1).

3. **Neutrophils.**

Neutrophil adhesion to cultured endothelium was, in general, lower than that of lymphocytes, and did not differ significantly between HUVEC and HEVC. In 4 experiments, neutrophils demonstrated 7.5±2.3% adherence to HUVEC, and 8.7±2.4% adherence to HEVC (Figure 7.1).
Figure 7.1. Adhesion of peripheral blood lymphocytes (PBL), CLL cells, and neutrophils to cultured HUVEC and HEVC. Cells were isolated and labelled as described, and static adhesion assays carried out as detailed in Special Methods in this chapter. Data are given as the percentage of adherent cells, and is the mean±SE of the number of experiments indicated on the respective histograms.
Effect of IL-1 on leucocyte adhesion to cultured endothelial cells

The effect of pretreating endothelial cells with IL-1 on lymphocyte binding was studied in 8 experiments. IL-1 treated HUVEC displayed enhanced adhesiveness for PBLs, the percentage of adherent cells rising from 18.5±0.9% to 44.3±2.4% (244±20% of baseline, p<0.0005, n=8, Figure 7.2a). Pretreating HUVEC with IL-1 (10 U/mL for 4 hours) also significantly enhanced the adherence of CLL cells (22.5±3.4% adhesion to IL-1 treated HUVEC, as compared with 16.3±2.9% adhesion to unstimulated HUVEC, p < .005, n=21, Figure 7.2a). However, the binding of CLL cells to IL-1 treated HUVEC is significantly lower than that of PBL to IL-1 activated HUVEC (p<.005), suggesting that under circumstances of endothelial activation, CLL cells may not be fully competent to bind to vascular endothelium.

In contrast, IL-1 treatment of HEVC had no effect on the binding of PBL (34.3±6.4% adherence to IL-1 treated HEVC, as compared with 33.0±6.8% adherence to untreated HEVC, Figure 7.2b). Similarly, the binding of CLL cells to HEVC is not affected by pretreating the endothelial cells with IL-1.

Neutrophils also demonstrated enhanced adhesion to IL-1 treated HUVEC, the percentage of adherent cells rising from 7.5±2.3% with untreated HUVEC to 30.2±7.0% with cytokine activated HUVEC (an increase to 500±135% of basal levels, p<.01, n=4, Figure 7.3). Once again, IL-1 had little consistent effect on neutrophil adhesion to HEVC. Neutrophil adhesion to IL-1 activated HEVC was 11.5±1.0%, as compared with 8.7±2.4% to untreated HEVC (n=4, Figure 7.3). This differential effect of IL-1
on the leucocyte binding properties of HUVEC and HEVC is thus seen with both lymphocytes and neutrophils.
Figure 7.2. Effect of IL-1 on the adhesion of PBL and CLL cells to (a) HUVEC and (b) HEVC. Confluent endothelial monolayers were incubated with IL-1 (10 U/ml for 4 hours) or medium as control, washed once with medium, and then used for lymphocyte adhesion assays as detailed above. Data is given as percentage (%) of adherent cells, and is the mean±SE of 8 experiments on PBL, 21 experiments on CLL adhesion to HUVEC, and 9 experiments on CLL binding to HEVC.
Figure 7.3. Effect of IL-1 on the adhesion of peripheral blood neutrophils to HUVEC and HEVC. Confluent endothelial monolayers were treated with IL-1 as detailed above. Neutrophils were isolated and labelled as described. Data is given as the percentage (%) of adherent cells, mean±SE of 4 experiments.
**Effect of interferon-alpha on leucocyte adhesion to cultured endothelial cells**

The effect of interferon-alpha on cell binding was investigated by studying the direct effects on both lymphocytes and endothelial cells. Pretreating CLL cells with interferon-alpha (500 U/mL for 18 hours) does not significantly alter cell binding to either HUVEC or HEVC monolayers (the binding of untreated CLL cells to HUVEC and HEV was 16.3±2.9% and 25.7±7.3% respectively, while that of interferon-alpha treated cells was 16.6±3.4% and 17.7±4% respectively, Figures 7.4a, b). Similarly, pretreating PBL with interferon-alpha had no effect on adhesion to either HUVEC or HEVC.

To confirm and extend these observations, experiments were carried out to examine the binding of CLL cells to frozen sections of lymphoid tissue. Human lymphoid sections did not support reliably reproducible levels of lymphocyte binding, as has been previously noted by other workers (Butcher et al, 1980). Monkey lymphoid sections were found to work better in this assay. In 5 experiments using monkey lymph node sections, the binding of interferon-alpha treated CLL cells was 93.9±18.0% of the binding seen with untreated cells. However, the level of nonspecific binding of lymphocytes was still unacceptably high. Figure 7.5 shows a representative experiment with lymphocytes adhering to the luminal surface of a high endothelial venule in cross section.

Pretreating endothelial cells (both HUVEC and HEVC) with interferon-alpha (500 U/ml for 18 hours) also had no effect on the binding of either CLL or PBL, whether untreated or interferon-alpha treated.
Legend Figure 7.4.
Lymphocytes were preincubated with interferon-alpha (500 U/mL for 18 hours) or medium as control, washed 3 times with medium, then labelled with $^{51}$Cr and used in adhesion assays as detailed under *Special Methods*. Confluent endothelial monolayers in 96 well tissue culture plates were incubated with IL-1 (10 U/mL for 4 hours) or medium as control and washed once in warm fresh medium prior to being used in adhesion assays. Data is given as percentage (%) of adherent cells, mean±SE.
(a) Adhesion of PBL (n=6) and CLL cells (n=21) to HUVEC.
(b) Adhesion of PBL (n=6) and CLL cells (n=8) to HEVC.
Figure 7.4. Effect of interferon-alpha on the adhesion of PBL and CLL cells to (a) HUVEC and (b) HEVC.
Figure 7.5. Binding of CLL cells to lymphoid tissue sections. The frozen section binding assay was carried out using CLL cells preincubated with interferon-alpha (500U/mL), and shows lymphocytes adhering to the cut surface of a high endothelial venule.
CD18 dependence of lymphocyte binding to HEVC and HUVEC

In 1 experiment, functional inhibition studies were carried out to examine the degree to which lymphocyte binding was dependent upon CD18 mechanisms. 8 replicate wells were used for each data point. MAb MHM23 (anti-CD18) significantly reduced the adhesion of PBL to unstimulated HUVEC (from 55.2% to 12.3%, i.e. 78% reduction in adhesion, Figure 7.6a). CD18 also appeared to mediate the binding of lymphocytes to HEVC, but to a lesser extent; the percentage of adherent cells was reduced from 72.2% to 43.1% (40% reduction) by mAb MHM23 (Figure 7.6b). In contrast, the enhanced adherence to IL-1 activated HUVEC was much less dependent on CD18 (% adhesion of lymphocytes to IL-1 activated HUVEC is reduced from 80.2% to 63.4% by mAb MHM23). In fact, the presence of anti-CD18 mAb appears to enhance the effect of IL-1 on lymphocyte adhesion to HUVEC (adhesive increment produced by IL-1 in the presence of mAb MHM23 is 51.1%, while that in the absence of mAb MHM23 is only 24.8%). The effect of anti-CD18 mAb on the adherence of lymphocytes to HEVC is independent of the conditions of endothelial activation (Figure 7.6b).
Legend Figure 7.6
Lymphocytes and confluent endothelial monolayers were prepared as detailed above. Anti-CD18 mAb (10 μg/mL), or an isotype matched control antibody, was added to lymphocyte suspensions at the start of the adhesion assay, and was present throughout. Data is mean±SD of quadruplicate samples in 1 experiment, and is given as percentage (%) of adherent cells.
Figure 7.6. Effect of mAb MHM23 (anti-CD18) on lymphocyte adhesion to (a) HUVEC, and (b) HEVC.
DISCUSSION

The aims of the work presented in this chapter were two-fold. The first was to develop and characterize an in vitro culture system for human high endothelial cells, which could be used to study the adhesive mechanisms involved in the homing of both normal, and CLL cells. The second aim of these studies was to investigate the adhesion of B-CL cells to vascular endothelium, using both high endothelial cell cultures, and HUVEC, in the context of cytokine stimulation. In particular, I was concerned to discover if the induction of L-selectin expression by Interferon-alpha correlated with increased cell binding to endothelium.

Human tonsillar high endothelial cells were characterized and identified by the demonstration of high uptake of radioactive sulphur, high levels of expression of endothelial adhesion molecules, and the preferential binding of lymphocytes, when compared with human umbilical vein derived endothelial cells (HUVEC). High endothelial cells in rats synthesize a unique sulphated glycolipid, and the rapid preferential uptake of $^{35}$S by these cells has been used to identify high endothelial cells in lymphoid tissue (Andrews et al, 1980). Similarly, incubation of slices of rat popliteal lymph node with $^{35}$S led to the selective labelling of high endothelium while other cell types demonstrated no uptake. Endothelial cells isolated from human tonsils in this study demonstrated highly selective uptake of $^{35}$S, at approximately 6 times that of HUVEC, a similar magnitude of uptake to that shown for rat lymph node HEVC (Ise et al 1988).

The absence of vWF antigen expression by cultured HEVC in this system confirms other reports in which immunofluorescent
labelling of tissue sections of rat lymph nodes failed to demonstrate any vWF antigen staining on the specialised post-capillary venules in the paracortex of cervical lymph nodes, while the endothelium lining other vessels, such as the aorta, the hilar arteries and vein, were positive. The same studies also found primary HEVC cultures to be negative for vWF Ag expression. In another study, cultured HEVC obtained from rat Peyer's patches also failed to stain for vWF Ag (Chin et al 1990). Lack of vWF expression appears to be a characteristic of endothelium lining HEV in vivo, as well as of cultured HEVC in vitro.

Cultured human HEVC expressed high basal levels of adhesive ligands, an observation which is consistent with the view that high endothelium is specialized to support lymphocyte binding and migration. ELAM-1 is an endothelial-specific inducible adhesion receptor which mediates leucocyte adhesion to cytokine activated endothelium (Shimizu et al 1991), while ICAM-1 and VCAM-1, although not specific for endothelial cells, are upregulated on endothelium following stimulation by cytokines, and also mediate leucocyte adhesion. Enhanced expression of these endothelial surface adhesion receptors has also been demonstrated in inflamed tissues in vivo (Cotran et al 1986, Rice et al 1991), in the context of leucocyte infiltration. The relationship between high levels of surface adhesion molecules and the possession of an HEV phenotype has recently been explored in immunohistochemical studies. High endothelium is a morphologically and functionally unique type of endothelium lining venules in the T cell areas of lymph nodes and mucosa-associated lymphoid tissues (Kraal et al 1986). The expression of high endothelial phenotype and function is not a constant feature,
but is under dynamic regulatory influences, for eg., lymph nodes deprived of afferent lymphatics lose their cuboidal shape and become flat, and lose the ability to sustain lymphocyte migration (Hendriks et al 1980, Hendriks et al 1987). Antigenic stimulation is thought to play a part in sustaining high endothelial morphology and function, an hypothesis which is supported by the fact that persistent antigenic stimulation leads to the development of HEV-like vessels at inflammatory sites outside lymphoid tissue (Freemont & Ford 1985). HEVC in chronic inflammatory sites display enhanced adhesiveness for lymphocytes (Jalkanen et al 1987), and increased expression of ELAM-1 and VCAM-1 (Koch et al 1991). The enhanced expression of VCAM-1 has been shown to be directly involved in lymphocyte binding to synovial HEV in rheumatoid arthritis (Dinther-Janssen et al 1991). In this chapter, human tonsillar derived cultured HEVC express high basal levels of the endothelial surface adhesion receptors, ICAM-1, ELAM-1 and VCAM-1, a property now generally accepted as being associated with endothelium that has become activated, or specialized to support leucocyte adhesion and extravasation, both in vitro, and in vivo. In contrast, HUVEC expressed comparatively low levels of endothelial adhesive ligands, supporting the view that these large vessel derived endothelial cells are relatively undifferentiated and non-specialized.

The already high basal expression of adhesion receptors on HEVC was not further upregulated by IL-1, suggesting, either that these molecules are already maximally expressed on HEVC, or that cultured HEVC do not respond to IL-1 stimulation under these conditions. In contrast, IL-1 treated HUVEC displayed increased
levels of ICAM-1, ELAM-1 and VCAM-1, in accord with previous reports (Pober et al, 1986, Bevilacqua et al, 1987, Osborn et al, 1989). The results on human tonsillar HEVC presented here are in keeping with a previous report that HEVC isolated from rat lymph node expressed high levels of ICAM-1 and that IL-1 stimulation for up to 24 hours had no effect on these levels (May & Ager 1992). Hence HEVC cells may differ from large vessel endothelial cells in the response to certain cytokines, and in the utilization of different adhesive pathways for leucocyte binding.

Finally, cultured HEVC were characterized by the high levels of preferential (over HUVEC) binding by lymphocytes. Neutrophils also demonstrated specific binding to HEVC, but at levels comparable to the adhesion to HUVEC. This is the first direct comparison of the leucocyte binding capabilites of HEVC and HUVEC. A previous study has shown that lymphocytes demonstrate specific binding to cultured rat HEVC, as compared with binding to fibroblasts and aortic endothelial cells (Ager & Mistry 1988). These observations, in turn, confirm previous \textit{in vitro} work using the frozen section assay, as well as histological studies which show that high endothelial differentiation in nonlymphoid tissue is almost exclusively found in the context of extensive lymphocytic infiltrates. The binding of neutrophils to cultured HEVC confirms and extends previous reports of neutrophil binding to HEV in frozen lymph node sections (Lewinsohn \textit{et al} 1987, Jutila \textit{et al} 1989). The lack of preferential neutrophil adhesion to HEVC, despite the high levels of endothelial adhesive ligands on high endothelium is intriguing, and may relate to the fact that, while lymphocytes extravasate
via HEV into lymphoid tissue in vivo, neutrophils do not, as a rule, migrate into lymph nodes.

Leucocyte adhesion to cultured human HEVC was not altered by IL-1 stimulation, this observation is consistent with the unchanged levels of adhesion receptors, and confirms previous work which showed, using cultured Peyer's patch HEVC (rat), that TNF pretreatment of HEVC increased lymphocyte adhesion, whereas IL-1 was without effect (Chin et al, 1990) Similarly, in a separate study using HEVC derived from rat cervical lymph nodes, an effect of IL-1 on lymphocyte adhesion was only observed after 24 hours, and was modest when compared with the effect of TNF, or of interferon-gamma (May & Ager, 1992). In contrast, IL-1 stimulation of HUVEC enhances the binding of both lymphocytes and neutrophils, as has been previously reported (Smith et al 1991). Taken together, these results are consistent with the data on adhesion molecule expression and suggest, either that the endothelial adhesive mechanisms upregulated by IL-1 on HUVEC may already be maximally expressed on HEVC, or that HEVC have a limited capability of responding to IL-1.

The involvement of CD18 dependent pathways in lymphocyte adhesion to HEV has been previously demonstrated using the frozen section assay (Pals et al 1989), and, more recently, using cultured HEVC derived from rat peripheral LN (Tamatani et al 1991). The results here, using cultured human high endothelium confirm and extend these observations. LFA-1 is considered to function as an accessory adhesion molecule in lymphocyte homing, augmenting lymphocyte adhesion to HEV, but without imparting organ-selectivity. The involvement of LFA-1 dependent adhesive pathways in lymphocyte adherence to HUVEC is in accord
with previous reports (Haskard et al 1986), as is the lesser dependence of lymphocyte adhesion to cytokine activated endothelium. This could be explained by the engagement of other adhesion pathways, such as the binding of lymphocyte surface VLA-4 to VCAM-1, which is upregulated on IL-1 activated endothelium.

CLL cells demonstrate higher levels of binding to HEVC than to HUVEC, and are also able to upregulate adhesion to IL-1 treated HUVEC, but not to the extent seen with normal PBL. The adhesion of CLL cells to IL-1 activated endothelium is significantly lower than that of PBL. Cytokine stimulation of endothelium appears to highlight a particular defect in CLL cell interaction with endothelium, and this may relate to the relatively low levels of the leucocyte integrin, CD11a (LFA-1) on CLL cells. The ligand for CD11a is ICAM-1, which is upregulated on cytokine-stimulated endothelium, and the LFA-1/ICAM-1 pathway mediates a substantial part of the enhanced adhesion seen with normal PBL (Haskard et al, 1986). In general, CLL cells demonstrate a similar dependence on CD18 in binding to unstimulated endothelium, suggesting that these malignant cells are able to utilize some adhesive mechanisms of normal lymphocytes in interacting with endothelium.

Interferon-alpha has no effect on the binding of either CLL cells or normal lymphocytes to HEVC or HUVEC, despite the fact that, in parallel experiments, the same CLL cells demonstrated an increase in L-selectin expression in response to interferon-alpha stimulation. In addition, when applied to endothelial cells, interferon-alpha has no effect on surface expression of adhesive receptors, or on the adhesivity of these cells for lymphocytes.
There are some possible explanations for the lack of increased cell binding despite the upregulation of L-selectin. There has been one report of an abnormal L-selectin molecule on CLL cells, this molecule had a lower Mr than the L-Selectin receptor from normal lymphocytes, and L-selectin RNA from CLL cells displayed faster electrophoretic mobility than that from normal lymphocytes, suggesting that the L-selectin receptor in CLL may be qualitatively abnormal (Prystas et al. 1992). These studies have not been confirmed by others, in particular, another group has found that the L-selectin receptor on CLL cells migrated in a similar fashion to the L-selectin receptor precipitated from normal lymphocytes (Spertini et al. 1991). This same group also found the L-selectin receptor on CLL cells to be competent in mediating the binding of CLL cells to HEV in the frozen section assay, the degree of binding correlating with the levels of L-selectin expression, and this binding was inhibited by anti-L-selectin antibody. Another point to make here is that the static in vitro adhesion assay employed in this study may not be comparable to the in vivo situation, where haemodynamic forces, cell deformability and vessel size all play a part in the adhesive interactions of circulating cells with endothelium. The L-selectin receptor, like the other selectin molecules, is thought to mediate leucocyte adhesion to endothelium mainly under conditions of flow (Lawrence & Springer 1991). It is noteworthy that the initial characterization of L-selectin as the peripheral lymph node homing receptor was done using the frozen section assay, in which lymphocytes laid on lymph node sections are kept in constant rotatory motion (Stamper & Woodruff 1976). In addition, a recent study has shown that L-selectin participates in
the adhesion of both lymphocytes and neutrophils to TNF-activated HUVEC only under rotating (non-static) conditions (Spertini et al 1991).

In summary, cultured human tonsillar high endothelial cells provide a suitable *in vitro* system for the study of lymphocyte binding to this specialized endothelium. The impaired binding of CLL cells to IL-1 activated endothelium suggests that these malignant cells may be defective in certain aspects of adherence dependent immune functions such as localisation into inflammatory areas *in vivo*. On the other hand, the demonstration of preferential binding to cultured high endothelium suggests that CLL cells may be able to exhibit normal homing behaviour *in vivo*, thus accounting for the known patterns of spread and tissue involvement in this disease. These results also suggest that malignant and normal cells use the same adhesive pathways in interacting with vascular endothelium, but may differ in their ability to maximize particular adhesive mechanisms in certain situations, such as the binding to inflamed endothelium. Finally, interferon-alpha had no direct effect on the binding properties of either lymphocytes or endothelial cells.
CHAPTER 8

EXPRESSION OF THE \textit{bcl-2} GENE PRODUCT IN CLL CELLS:
RELATIONSHIP TO APOPTOSIS, AND THE EFFECTS OF
CYTOKINE STIMULATION

INTRODUCTION

Among the many diverse effects of interferon-alpha is the
regulation of gene expression in both normal and malignant cells.
The induction of proteins associated with the anti-viral state,
such as the (2'-5')-oligoadenylate synthetase and the Mx protein
in the mouse have been extensively studied and reviewed
(Baglioni 1979, Lengyel 1982, Pestka \textit{et al} 1987, Staeheli &
Haller 1987). Interferon-alpha has also been reported to regulate
the expression of several cellular oncogenes in tumour cells
(Jonak & Knight 1987, De Maeyer & De Maeyer-Guignard 1988), for
example \textit{c-myc} (Kimchi 1987), \textit{c-fos} (Einat \textit{et al} 1985), and \textit{c-}
\textit{Ha-ras} (Samid & Friedman 1986). The products of these genes
appear to be involved in the control of cell growth and
proliferation, acting, for example, in signal transduction
pathways (eg \textit{ras}) or as nuclear transcription factors (eg \textit{myc}).
Recently a new category of proto-oncogenes has been identified,
which are involved in the control of programmed cell death, such
as p53 and \textit{bcl-2} (Korsmeyer 1992).

The \textit{bcl-2} gene was initially identified because it is
involved in the t(14;18) chromosomal translocation that occurs in
the follicular lymphomas (Tsujimoto \textit{et al} 1985a). This
translocation is the commonest in lymphoid malignancies,
occurring in approximately 85% of follicular lymphomas and 20%
of diffuse lymphomas (Fukuhara et al 1979, Yunis et al 1987), and results in the bcl-2 gene at 18q21 becoming juxtaposed with the enhancer of the immunoglobulin heavy chain genes at 14q32 (Tsujimoto et al 1985b, Bakhshi et al 1985, Cleary & Sklar 1985). This produces marked deregulation of the bcl-2 gene, and leads to inappropriately raised levels of the bcl-2-immunoglobulin fusion mRNA (Cleary et al 1986, Graninger et al 1987, Seto et al 1988). Most breakpoints occur in the untranslated region of the bcl-2 gene, so that both normal and translocated alleles produce the same 25kD protein (Tsujimoto et al 1986, Tsujimoto et al 1987). Haematopoietic cell lines transfected with a bcl-2-immunoglobulin mini-gene product demonstrated prolonged survival after growth factor withdrawal (Vaux et al 1988, Nunez et al 1990), and this appears to be due to interference with the process of programmed cell death or apoptosis (Hockenbury et al 1990). Apoptosis is an active process, distinct from necrosis, and is important in embryological and immunological development (Cohen 1991, Ellis et al 1991, Cohen et al 1992). It is characterised by the condensation of the cytoplasm and nucleus, fragmentation of the nucleus and cleavage of the DNA into oligosomal fragments, and finally fragmentation of the cell into membrane bound bodies (Wyllie et al 1980).

Transgenic mice expressing the bcl-2-immunoglobulin mini-gene demonstrate an accumulation of polyclonal, small resting B-cells that express IgM/IgD, 97% of which are in Go/G1 of the cell cycle (McDonnell et al 1989, McDonnell et al 1990). Interestingly, chronic lymphocytic leukaemia is characterised by the accumulation of monoclonal, small resting
B-cells that express IgM/IgD, and 97% of which are in Go/G1 of the cell cycle (Freedman 1990, Foon et al 1990, Dighiero et al 1991). CLL cells, however, only rarely express rearrangement of the \textit{bcl-2} gene (Raghoebier et al 1991, Mariano et al 1992), although they do express bcl-2 protein (Pezzella et al 1990, Zutter et al 1991).

This chapter examines the level of expression of bcl-2 protein in CLL cells, the role of the t(14;18) translocation in CLL, the effect of alpha-interferon on bcl-2 expression, and the role of bcl-2 protein expression in mediating protection against apoptosis in the context of cytokine stimulation.
SPECIAL METHODS

Immunophenotyping
E-rosette-negative cells were isolated from patients with CLL at various stages of the disease, and from patients with Binet stage A disease prior to, and after starting on interferon-alpha therapy. Cytocentrifuge preparations were made and stained using the APAAP method as described in Chapter 2.

For indirect immunofluorescence, CLL cells were incubated for 18 hours in the presence or absence of interferon-alpha (500U/mL), and then permeabilized in 70% ethanol for 10 minutes at -20°C. Cells were then stained and analysed on the EPICS flow cytometer as described in Chapter 2.

Cell treatment
For studies on apoptosis, CLL cells were incubated for 18 hours in the presence or absence of interferon-alpha (500U/mL). Cells were induced to apoptose either by co-incubating with hydrocortisone ($10^{-5}$M), or by prior exposure to 1500 rads of gamma radiation. In some experiments, cycloheximide was included at 50μg/mL. All incubations were carried out at 37°C.

DNA electrophoresis
CLL cells incubated for 18 hours in the presence or absence of interferon-alpha (500U/mL) were lysed in 0.5mL lysis buffer (10mM Tris, 0.1mM EDTA, 100mM sodium chloride, 1% sodium dodecyl sulphate, pH 8.0), and incubated overnight with 200μg/mL proteinase K. 0.5mL of phenol/chloroform (1:1) was added to the tubes, followed by gentle mixing for one hour. After
centrifugation at 13,000g for 10 minutes, the aqueous phase containing the DNA was harvested, and the DNA precipitated overnight at -20°C in 1mL absolute ethanol containing 50μL of 3M sodium acetate. The precipitates were pelleted at 13,000g for 10 minutes, air dried and redissolved in 10mM Tris, 0.1mM EDTA. Electrophoresis was carried out in a 1% agarose gel, and DNA was visualized with ethidium bromide.

**DNA fragmentation assay**
The extent of DNA fragmentation was determined by a modification of the diphenylamine reaction (Burton 1955) used by Cohen and Duke (Cohen & Duke 1984). Cells were lysed in 0.4mL lysis buffer (10mM Tris, 1mM EDTA, 0.2% Triton X-100, pH 7.5), and the lysates were centrifuged at 13,000g for 10 minutes to separate intact chromatin from fragmented DNA. Pellet and supernatant were separated and precipitated overnight at 4°C in 12.5% trichloroacetic acid. After sedimentation at 13,000g for 4 minutes, the precipitates were hydrolysed in 80μL trichloroacetic acid at 90°C for 10 minutes. 0.16mL of diphenylamine reagent (0.15g diphenylamine, 0.15mL sulphuric acid, 0.05mL acetaldehyde (16mg/mL stock)/ 10mL acetic acid) was added to each tube, and colour allowed to develop overnight. 0.2mL of each sample were transferred to a 96 well flat bottomed plate, and the absorbance read at 570nm on a microplate reader (Dynatech labs). The percentage DNA fragmentation was calculated from the ratio of DNA in the supernatant to the total DNA in the supernatant and in the pellet.
Polymerase chain reaction

DNA was prepared as described above for DNA electrophoresis, and a semi-nested polymerase chain reaction (PCR) was used to detect the t(14;18) chromosome translocation. The method and primers were kindly provided by Tim Diss in the Department of Histopathology, University College and Middlesex School of Medicine, London. 30 cycles were carried out using 200ng DNA with primers 693 and 943, followed by 20 cycles with 1μL of first round product and primers 693 and 942. Primers 942 and 943 are overlapping consensus sequences from the joining region of the immunoglobulin heavy chain gene on chromosome 14 (Trainor et al 1990) and primer 693 is at the 5' end of the major breakpoint region of bcl-2 on chromosome 18 (Crescenzi et al 1988). Amplifications were carried out in PCR mixtures of 10mM Tris-HCl pH 8.3, 50mM KCl, 9mM MgCl$_2$ (3.5mM in the second round), 0.001% gelatin, 200μg each dNTP, and 250ng of each primer, in a total volume of 100μL. The samples were heated to 96°C for 7 minutes, cooled to 61°C before the addition of 1 unit of Taq polymerase. The PCR was carried out in a thermal cycler using a programme of 40 seconds at 93°C, 45 seconds at 61°C, and 110 seconds at 72°C. A final extension step of 5 minutes at 72°C was allowed. A positive control was included from a patient with a follicular lymphoma expressing the t(14;18) translocation. PCR products were analysed on 3% agarose gels and the DNA visualized with ethidium bromide.
RESULTS

**CLL cells express bcl-2 protein**

CLL cells from 19 patients at various stages of disease were examined. Cytocentrifuge preparations were stained with anti-bcl-2 antibody and anti-CD5 antibody, and visualized using the APAAP method. Slides were scored on a comparative basis according to the following criteria: negative (-), weakly positive (+), moderately positive (++), strongly positive (+++). All CLL cells expressed both bcl-2 protein and CD5 with a uniform distribution (Table 1). No significant association was detected between bcl-2 protein expression and Binet stage of disease.

**Expression of bcl-2 protein in CLL is not dependent on rearrangement of the bcl-2 gene.**

DNA was prepared from cells taken from twelve patients with CLL at various stages of disease, and from one patient with follicular B-cell lymphoma expressing the t(14;18) chromosome translocation. The DNA was then amplified in a semi-nested polymerase chain reaction (PCR) using probes specific for the t(14;18) translocation. PCR product from the DNA prepared from the patient with follicular B-cell lymphoma run on agarose gels showed the predicted band of approximately 220 base pairs (Figure 8.1). In none of the PCR products from the DNA prepared from the CLL patients was the specific product demonstrated (Figure 8.1)
Table 8.1. Expression of bcl-2 protein and CD5 in cells from patients with CLL at various Rai stages of disease.

E-rosette negative cells were stained using the APAAP technique. Cells were scored as follows; negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++).
Figure 8.1. CLL cells do not express the t(14;18) translocation.

PCR products from DNA of 8 patients with CLL (lanes 2-9), and 1 patient with follicular lymphoma (lane 10). Lane 1 shows DNA standards.
Interferon-alpha therapy increases expression of bcl-2 protein in CLL cells in vivo.

Eight patients with Binet stage A CLL were studied. Cytocentrifuge preparations of E-rosette negative cells obtained prior to starting on interferon-alpha therapy, and at various times afterwards (mean 4 weeks, range 3-6 weeks) were stored at -70°C. Pre- and post-therapy slides from each patient were then stained in parallel for bcl-2 protein and CD5 expression using the APAAP method as outlined in Chapter 2. Slides were scored as above. Interferon-alpha increased expression of bcl-2 protein in all eight patients studied (Figure 8.2a,b), while CD5 expression did not change significantly (Figure 8.2c,d).

Interferon-alpha increases expression of bcl-2 protein in CLL cells in vitro.

The effect of interferon-alpha treatment in vitro on the expression of bcl-2 protein was examined in ten patients with CLL at various stages of disease. E-rosette negative cells from were incubated for 18 hours in the presence or absence of interferon-alpha (500U/mL), permeabilized in 70% ethanol for 10 minutes at -20°C, and then stained by indirect immunofluorescence and analyzed on the EPICS flow cytometer as described in Chapter 2. Cells incubated without interferon-alpha were 79.7±8.4% positive for the bcl-2 protein, and the mean cell fluorescence (MCF) was 24.8±6.2. Cells incubated with interferon-alpha were 94.6±3.2% positive (p<.01, n=10) with an MCF of 49.6±5.8 (p<.001). Figure 8.3 shows the flow cytometry profile for 1 representative patient. Unpermeabilized cells showed no specific staining for bcl-2 protein.
Figure 8.2 (a,b). Interferon-alpha increases expression of bcl-2 protein in CLL cells in vivo.
Cytocentrifuge preparations obtained from 1 patient prior to and 4 weeks after starting on interferon-alpha, were stained for bcl-2 protein using the APAAP technique. (a) before treatment, (b) after 4 weeks of therapy.
Figure 8.2 (c,d). Interferon-alpha has no effect on the expression of CD5 in CLL cells.
Duplicate cytocentrifuge preparations were stained for CD5, as a control antigen, (c) prior to, and (d) 4 weeks after starting on interferon-alpha, in the same patient.
Figure 8.3. Pretreatment of CLL cells with interferon-alpha increases expression of bcl-2 protein *in vitro*.

CLL cells preincubated with interferon-alpha (500U/mL for 18 hours at 37°C), or medium as control, were stained by indirect immunofluorescence and analysed by flow cytometry. Panel a, control, panel b, interferon-alpha treated cells. The cursor shown in each panel denotes the gate set such that 5% of cells in the negative sample stained with isotype-matched antibody were positive.
Interferon-alpha protects CLL cells against apoptosis in vitro.

The effect of interferon-alpha on apoptosis in vitro was examined in ten patients with CLL at various stages of disease. DNA prepared from CLL cells cultured for 18 hours in medium alone showed the typical cleavage of DNA into oligonucleosomal fragments and the characteristic 'DNA ladder' when run on agarose gels (Figure 8.4). Co-incubation with hydrocortisone (10^{-5}M), or exposure of the cells to 1500 rads of gamma radiation before incubation increased the level of DNA fragmentation. The presence of interferon-alpha in the incubation mixtures reduced the level of DNA fragmentation under all conditions (Figure 8.4). This effect was quantitated using the diphenylamine reaction (Figure 8.5). CLL cells incubated in medium alone showed 35.6±10.3% DNA fragmentation. In the presence of interferon-alpha this was decreased to 6.6±5.8% (p<.0001, n=10). Incubation with hydrocortisone (10^{-5}M) increased DNA fragmentation to 63.9±12.6% (p<.01), but, in the presence of interferon-alpha, this was decreased to 10.8±4.5% (p<.001). Similarly, exposure of the cells to 1500 rads of gamma radiation before incubation increased the level of DNA fragmentation to 80.0±2.9% (p<.001), but, in the presence of interferon-alpha this was decreased to 5.4±1.6% (p<.0001, Figure 8.5). The protective effect of interferon-alpha on hydrocortisone induced apoptosis was dose-dependent with optimal activity between 500U/mL and 1000U/mL (Figure 8.6). This protective effect was maintained for up to 24 hours (Figure 8.7).
Figure 8.4. Interferon-alpha protects CLL cells against apoptosis in vitro.

DNA was prepared from CLL cells were pretreated for 18 hours under the following conditions as denoted below. Lane 1, DNA standard markers; lane 2, medium only; lane 3, interferon-alpha (500U/mL); lane 4, hydrocortisone (10^{-5}M); lane 5, interferon-alpha and hydrocortisone, and lane 6, 1500 rads gamma-irradiation.
Figure 8.5. The effect of interferon-alpha on spontaneous and induced DNA fragmentation. Cells were preincubated for 18 hours under the following conditions, and DNA fragmentation was quantitated using the diphenylamine reaction as outlined in Special Methods. Data are mean±SE of 10 experiments.

NIL  medium alone (control)
IFN  500U/mL interferon-alpha
HC   10^{-5} M hydrocortisone
HC+IFN 10^{-5} M hydrocortisone plus 500U/mL interferon-alpha
IRR  1500 rads gamma radiation
IRR+IFN 1500 rads gamma radiation plus 500U/mL interferon-alpha
Figure 8.6. Dose dependent effects of interferon-alpha and hydrocortisone on DNA fragmentation. CLL cells were pretreated with interferon-alpha and hydrocortisone at various doses in combination, and DNA fragmentation determined using the diphenylamine reaction. Data are the means of duplicate determinations in an experiment on a representative patient.
Figure 8.7. The effect of interferon-alpha on hydrocortisone induced apoptosis over time.

Cells were analysed for DNA fragmentation using the diphenylamine reaction, at various times following the completion of 18 hours incubation with interferon-alpha. Results are mean of duplicate determinations from 1 representative patient.

HC \quad 10^{-5} \text{M hydrocortisone}

HC+IFN \quad 10^{-5} \text{M hydrocortisone plus 500U/mL interferon-alpha}
The protective effect of interferon-alpha on apoptosis of CLL cells is dependent on protein synthesis

The effect of the protein synthesis inhibitor, cycloheximide, on apoptosis and the protective effect of interferon-alpha was examined in cells from 5 patients with CLL. CLL cells were incubated for 18 hours either in medium alone, with interferon-alpha (500U/mL) alone, with hydrocortisone (10⁻⁵M) alone or with interferon-alpha and hydrocortisone. All conditions were repeated in the presence of 50μg/mL cycloheximide. CLL cells cultured in medium alone showed 34.3±8.7% DNA fragmentation, which was decreased to 3.3±2.6% when co-incubated with interferon-alpha (p<.0001, n=5). Hydrocortisone increased the level of DNA fragmentation to 58.6±9.4% (p<.01), and interferon-alpha was able to abolish this effect, reducing the level of DNA fragmentation to 15.8±7.6% (p<.001). Incubation with cycloheximide increased the level of both spontaneous DNA fragmentation, and that induced by hydrocortisone, and completely abrogated the protective effect of interferon-alpha (Figure 8.8). In the presence of cycloheximide (50μg/mL), DNA fragmentation was 47.3±11.4% without, and 54.8±12.3% with interferon-alpha. Co-incubation with cycloheximide and hydrocortisone produced DNA fragmentation levels of 69.2±10.4%, while the addition of interferon-alpha produced fragmentation levels of 64.8±14.2% (Figure 8.8).
Figure 8.8. The effect of cycloheximide on the protective effect of interferon-alpha on DNA fragmentation. CLL cells were treated for 18 hours under each of the following conditions, and DNA fragmentation analysed using the diphenylamine reaction. Values are mean±SD of 5 separate experiments on different patients.

NIL medium alone
IFN 500U/mL interferon-alpha
HC 10⁻⁵M hydrocortisone
HC+IFN 10⁻⁵M hydrocortisone plus 500U/mL interferon-alpha
CHX 50µg/mL cycloheximide
CHX+IFN 50µg/mL cycloheximide plus 500U/mL interferon-alpha
HC+CHX 10⁻⁵M hydrocortisone plus 50µg/mL cycloheximide
HC+CHX+IFN 10⁻⁵M hydrocortisone plus 50µg/mL cycloheximide plus 500U/mL interferon-alpha
DISCUSSION

It is now clear that expression of bcl-2 protein is important in the normal physiological control of many tissues including both B- and T-cell development (Korsmeyer 1992). In secondary germinal centres, the cells of the follicular mantle express high levels of bcl-2 (Pezzella et al 1990, Hockenbury et al 1991). In contrast, proliferating centroblasts do not express bcl-2. It has been suggested that in these cells somatic mutation occurs to give altered antigen receptors (MacLennan & Gray 1986). The centrocytes in the basal part of the light zone (that also express no bcl-2) result from these cells, and are immediately selected on the follicular dendritic cells. Those that do not have sufficient affinity for antigen, and are therefore not selected, die by apoptosis (Liu et al 1989). Bcl-2 expression returns in the apical potion of the light zone, where selection and maintenance of memory cells and/or plasma cells occurs. Both the small recirculating memory cell (Pezzella et al 1990, Hockenbury et al 1991), and plasma cells (Hamilton et al 1991) express bcl-2.

Although there is some debate about the normal cellular counterpart of CLL cells, the fact that CLL cells express bcl-2 suggests a number of phenotypic similarities with the B-lymphocytes of the follicular mantle. CLL cells are small mature looking lymphocytes, expressing surface IgM and IgD, that also express bcl-2 (Pezzella et al 1990, Zutter et al 1991) and CD23 (Sarfati et al 1990, Fournier et al 1992). CLL cells, however, also express CD5 which is not an antigen highly expressed on cells of the follicular mantle. However, a small number of CD5 expressing B-cells have been demonstrated in the
periphery of the germinal centre but not in normal bone marrow (Caligaris-Cappio et al 1982). Interestingly, studies on the immunoglobulin gene rearrangements in CLL cells have shown that there is little or no somatic mutation in the variable region genes (Kipps et al 1991), suggesting that they have not gone through a cycle of antigen driven proliferation (MacLennan & Gray 1986). This suggests that CLL cells correspond to an immature virgin B-cell. These virgin cells normally have a short life-span, but there does appear to be population of long-lived virgin B-cells which are the result of an antigen-independent mechanism (MacLennan & Gray 1986). It is tempting to suggest that this mechanism may be associated with the expression of bcl-2, and that CLL cells may result from the inappropriate expression of bcl-2 protein, leading to prolonged survival and accumulation of CLL cells in the peripheral blood. The inappropriate expression of bcl-2 protein may render the CLL cells resistant to clonal deletion by antigen (Nossal 1983) and may help to explain the prevalence of auto-antibodies and auto-immune phenomena in CLL (Foon et al 1990, Dighiero et al 1991). Furthermore, transgenic mice expressing the bcl-2-immunoglobulin minigene product demonstrate prolonged secondary immune responses and develop auto-immune phenomena (Korsmeyer 1992).

The factors involved in the regulation of bcl-2 expression in CLL cells are unclear, but the role of interferon-alpha is particularly intriguing. The levels of interferon in efferent lymph from virally activated lymph nodes may be 200 times more than unstimulated nodes (Trnka & Cahill 1980), and may lead to retention of lymphocytes in the lymph node (Kimber et al 1987, Hein & Supersaxo 1988). Lymphocytes are thus exposed to high
levels of interferon for prolonged periods, and may respond by inappropriate expression of bcl-2, leading to prolonged survival and possibly malignant transformation. These processes might also be important in the aetiology of the auto-immune phenomena associated with many viral diseases. Furthermore it has recently been shown that cells involved in the immune response to a virus may themselves become targets of the cell-mediated reponse (Zinkernagel 1992). Immunopathological destruction of lymphoid follicles may result in dysregulation of responses or lead to immunosuppression (Odermatt et al 1991). These responses lead to loss of primary but not secondary humoral reponses (Zinkernagel 1992). It is possible therefore that induction of bcl-2 expression by interferon-alpha may render cells resistant to apoptosis induced by cytotoxic T-cells (Cohen 1991, Cohen et al 1992), and allow maintenance of the humoral immune response despite the immunopathological cell-mediated cytotoxic effects. Interestingly infection of B-cells by Epstein-Barr virus (EBV) induces expression of CD23 (Thorley-Lawson & Mann 1985) and bcl-2 (Henderson et al 1991) and protects infected cells against apoptosis. These effects may be related to production of interferon-alpha by infected cells.

The high level of spontaneous apoptosis in CLL cells cultured in vitro (Collins et al 1989) has two important implications. Firstly, the fact that a high proportion of CLL cells die following culture for 18 hours demands re-evaluation of much of the work regarding the regulation of differentiation and proliferation of CLL cells in vitro (Drexler et al 1988). Secondly, the control of the level of apoptosis in vivo has important clinical and therapeutic implications. The fact that both
corticosteroids and gamma-irradiation can enhance apoptosis in vitro appears to vindicate their use as therapy. In contrast, the protective effects of interferon-alpha would at its worst suggest that its use in CLL is entirely inappropriate, and at best that it may help to explain the highly variable and unpredictable responses.

The role of cycloheximide in enhancing apoptosis was unexpected. Protein and mRNA synthesis are required for the induction of apoptosis in many cell systems, whether mediated by exposure to glucocorticoids or irradiation induced death of thymocytes, death of interleukin dependent cell lines, or activation induced death of T cell hybridomas (Cohen 1991). It is not clear what the new proteins are but they may be the endogenous endonucleases responsible for the cleavage of DNA into oligonucleosomal fragments, or factors which activate this enzyme. On the other hand, it is now clear that in many cell types, inhibition of protein synthesis induces apoptosis. These cell types include metamyelocytes (Cohen 1991), basal keratinocytes (McCall & Cohen 1991) and many cell lines, including HL-60 (Martin et al 1990). These cells already appear to have activated the 'death pathway' but this is held in check by proteins with short half lives. It is of interest here that splenic B-cells, which share many characteristics with CLL cells, contain large amounts of endogenous endonuclease in their nuclei (Cohen 1991). The role of bcl-2 expression in these cells remains to be elucidated.

In summary, the results presented in this chapter suggest that CLL cells are primed to undergo apoptosis, but may be protected by cytokine stimulation which can induce bcl-2
expression. The following chapter examines in detail the way in which cytokines regulate apoptosis and proliferation of CLL cells.

The action of interferon-alpha to increase bcl-2 and protect CLL cells from apoptosis could be a secondary effect mediated through the activation of mononuclear cells in these cultures, particularly as alpha-interferon activates monocytes \textit{in vivo} (See Chapter 5). The role of other cytokines in protecting CLL cells against apoptosis and in inducing proliferation is examined in the next chapter.
CHAPTER 9
CYTOKINES AS GROWTH AND MAINTENANCE FACTORS FOR CLL CELLS

INTRODUCTION

It was first suggested that neoplastic transformation in cells infected by transforming viruses was due to an autocrine mechanism, and was the result of co-expression of a growth factor and its receptor (Sporn & Todaro 1980). It is now clear that autocrine secretion of growth factors plays a role in the malignant transformation of a variety of cancers (Sporn & Roberts 1985, Cross & Dexter 1991). Furthermore, many oncogenes encode proteins that are either growth factors (eg sis), growth factor receptors (eg kit, fms), proteins involved in transduction of growth factor responses (eg src, ras, raf) or are transcription factors that mediate growth factor induced gene expression (eg jun, fos), (Cantley et al. 1991).

A number of cytokines are involved in the regulation of normal growth and differentiation of B-cells (Hirano & Kishimoto 1988, Kinkade et al 1989, Steel & Hutchins 1989, Callard 1990), and several of these have been proposed as potential autocrine growth factors in CLL. For example, it has been suggested that CLL cells produce IL-1 (Pistoia et al 1986, Scala et al 1986), IL-6 (Biondi et al 1989, Freeman et al 1989), TNF (Cordingley et al 1988, Hahn et al 1989, Schena et al 1992), and soluble CD23 (Sarfati et al 1990, Fourier et al 1992). However, there is still some dispute as to whether CLL cells can respond to these factors by proliferation and/or differentiation, and what role, if
any, these factors play in the pathophysiology of the disease. Interferons have also been implicated as potential growth and differentiation factors in CLL (Ostlund et al 1986), and may therefore interact with any of the above factors.

Interferons have a number of effects on gene expression, inducing expression of some genes (eg (2'-5')-oligoadenylate synthetase, genes of the major histocompatibility complex (MHC)), and suppressing others (eg ornithine decarboxylase, glutamine synthetase). Interferon-alpha therapy may therefore suppress cytokine production, leading to the disruption of autocrine growth loops, or alternatively may enhance cytokine production leading to differentiation and/or proliferation.

This chapter explores some of these questions, and examines the effect of interferon-alpha therapy on the levels of circulating cytokines in CLL in vivo, as well as the effects of these cytokines on the proliferation and apoptosis of CLL cells in vitro.
SPECIAL METHODS

Patients and sample collection
Eight patients with Binet stage A B-CLL were examined for levels of circulating cytokines. These patients were the same patients which were studied in Chapters 4 and 5. Venous blood samples were taken before starting therapy with recombinant interferon-alpha 2a (Roferon), and at various times during therapy. The blood was allowed to clot at room temperature, serum removed and stored in aliquots at -70°C until used in assays. All samples were thawed only once. For proliferation and apoptosis assays, a further 10 patients with CLL at various stages of disease were studied. Cells were collected and prepared as described in Chapter 2.

IL-1β assay
Levels of IL-1β were determined using an enzyme immunoassay (R&D Systems, USA). Serum samples were incubated in duplicate for 2 hours at room temperature in a microtitre plate precoated with a monoclonal antibody specific for IL-1β. The plate was washed, peroxidase conjugated polyclonal antibody against IL-1β added, and the incubation continued for a further two hours at room temperature. The plate was washed again before addition of substrate (tetramethylbenzidine with hydrogen peroxide). The reaction was stopped after 20 minutes by adding 2N sulphuric acid, and the optical density measured at 450nm with wavelength correction at 570nm, on an MR700 microplate reader (Dynatech Laboratories, UK). The concentration of IL-1β was calculated from a standard curve obtained using recombinant human IL-1β.
**IL-6 assay**
Levels of IL-6 were determined using an enzyme immunoassay (R&D Systems, USA) as described above for IL-1β.

**TNF-α assay**
Levels of TNF-α were determined using an enzyme immunoassay (R&D Systems, USA) as described above for IL-1β.

**Soluble CD23 assay**
Levels of soluble CD23 (sCD23) were determined in an enzyme immunoassay (T-cell Diagnostics, USA). Serum samples were added to wells in a microtitre plate coated with a monoclonal antibody specific for CD23, followed immediately by a second horseradish peroxidase-conjugated monoclonal antibody specific for a distinct epitope on CD23. Following incubation for 2^{1/2} hours at room temperature on a rotator, the plate was washed, and freshly prepared chromogen substrate was added (O-phenylenediamine). After 30 minutes incubation at room temperature, the reaction was stopped by adding 2N sulphuric acid, and the optical density measured at 490nm with wavelength correction at 570nm, on an MR700 microplate reader (Dynatech Laboratories, UK). The concentration of sCD23 was determined by reference to a standard curve.

**Proliferation assays**
E-rosette-negative cells from patients with B-CLL at various stages of the disease were plated in triplicate at 5x10^5 in microtitre wells, and incubated for various times in the presence or absence of various cytokines. DNA synthesis was determined by
pulsing the wells with 1µCi of $^{3}$H-thymidine for the final 16 hours of the incubation. The wells were harvested on to glass fibre filters using the cell harvester (Dynatech Laboratories), and radioactivity measured in the β-counter.

**DNA fragmentation assay**

The extent of DNA fragmentation was determined by a modification of the diphenylamine reaction (Burton 1955) used by Cohen and Duke (Cohen & Duke 1984), as described in the previous chapter.
RESULTS

Levels of circulating cytokines in CLL patients on interferon-alpha therapy

Levels of IL-1β, IL-6, TNF-α, and soluble CD23 (sCD23) were measured in 8 patients with Binet stage A CLL receiving interferon therapy (Table 9.1).

Levels of IL-1β were undetectable in the serum of four out of eight patients at all times tested. In three other patients, sporadic samples had low levels of IL-1 (4-12 pg/mL), but these levels did not correlate with lymphocyte count, or Interferon-alpha therapy. One patient, CLL 5, did have significant levels of IL-1β at all times tested, mean 40.5 pg/mL, range 22-70 pg/mL (normal range <3.9 pg/mL). This patient showed a good response to interferon-alpha therapy, with a rapid fall in the lymphocyte count. This response was lost when the patient developed anti-interferon antibodies, and was regained as the antibody titre fell. The IL-1β levels showed significant (p<0.005) correlation with the lymphocyte count in this patient (Figure 9.1).
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<th>Time (weeks)</th>
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<th>IL-1 (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
<th>sCD23 (U/mL)</th>
<th>anti-IFN (IBU/mL)</th>
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Table 9.1. Serum levels of cytokines and soluble CD23 in 8 patients with CLL who received interferon-alpha therapy. Patients are designated as in Chapter 3, except for patient CLL 14, who was not included in Chapter 3.
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<th>Lymph (10^9/L)</th>
<th>IL-1 (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>TNF-(\alpha) (pg/mL)</th>
<th>sCD23 (U/mL)</th>
<th>anti-IFN (BU/mL)</th>
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Table 9.1 (contd.) Serum levels of cytokines and soluble CD23 in CLL patients receiving interferon-alpha therapy.
Figure 9.1. Correlation of lymphocyte count, levels of IL-β and anti-interferon antibodies in one patient with CLL on interferon-alpha therapy.
Levels of IL-6 were low or undetectable in all patients, apart from occasional high readings of >300pg/mL, which could have been related to concurrent infection or inflammation. In one patient, CLL3, levels of IL-6 were high before treatment, and fell in parallel with the lymphocyte count. When interferon-alpha therapy was stopped for 1 year in this patient, the lymphocyte count rose but the levels of IL-6 remained low (Figure 9.2). In a further two patients who developed anti-interferon antibodies, the level of IL-6 correlated with the change in the lymphocyte count (Figure 9.3).

Levels of serum TNF-α were detected in all patients, and levels were low and variable. In three patients, CLL 2, CLL 11, CLL 14, no correlation was seen between TNF-α levels and the lymphocyte count. In three other patients, CLL 1, CLL 3, CLL 6, the levels of TNF-α were elevated before therapy and fell during interferon-alpha treatment. All three patients lost response at some time during treatment but, despite this, TNF-α levels remained low. In a further two patients who developed anti-interferon antibodies, serum levels of TNF-α showed a positive correlation with changes in the lymphocyte count (Figure 9.4).

Levels of sCD23 were detected in the serum of all patients, at high concentrations, ranging from 140 to 1900 U/mL (normal range 15-250 U/mL). In three patients with levels of sCD23 greater than 1000U/mL, there was no correlation with lymphocyte count. In the other five patients, levels of sCD23 showed a correlation with changes in the lymphocyte count (Figure 9.5). Figure 9.6 shows the levels of sCD23 in 2 patients who developed anti-interferon antibodies.
Figure 9.2. Correlation of lymphocyte count and serum IL-6 in one patient with CLL on interrupted interferon-alpha therapy
Figure 9.3 (a,b). Correlation of (a) lymphocyte count and serum IL-6 and (b) anti-interferon antibodies in patient CLL 5.
Figure 9.3 (c,d). Correlation of (c) lymphocyte count and serum levels of IL-6 with (d) anti-interferon antibodies in patient CLL 12.
Figure 9.4 (a,b). Correlation of (a) lymphocyte count and serum levels of TNFα, and (b) anti-interferon antibody in patient CLL 5 while on interferon alpha therapy.
Figure 9.4 (c,d) . Correlation of (c) lymphocyte count and levels of TNFα with (d) anti-interferon antibody titre in patient CLL 12 while on interferon alpha therapy.
Figure 9.5 (a). Correlation of lymphocyte counts with serum levels of sCD23 in patient CLL 3.
Figure 9.5(b,c). Correlation of lymphocyte counts with serum levels of sCD23 in two CLL patients on interferon-alpha therapy.
Figure 9.6 (a,b). Correlation of (a) lymphocyte count, serum sCD23, and (b) anti-interferon antibody levels in patient CLL 5 on interferon alpha therapy.
Figure 9.6 (c, d). Correlation of (c) lymphocyte count, serum sCD23, and (d) anti-interferon antibodies in patient CLL 12 on interferon alpha therapy.
Proliferative responses of CLL cells to cytokines in vitro

CLL cells were incubated with various cytokines either alone or in combination with Interferon-alpha. Concentrations of cytokines were selected which had been previously shown to have maximal activity in heterologous biological systems. Cultures were initially incubated for varying lengths of time, and then pulsed with 1μCi of ³H-thymidine over the last 16 hours. Preliminary data suggested that 4 day incubation was optimal for most responses (Figure 9.7) and therefore the bulk of the experiments were harvested at that point. No significant ³H-thymidine incorporation was seen when CLL cells were incubated with 500U/mL interferon-alpha alone or in combination with TNF-α (500U/mL), IL-1β (10U/mL), IL-4 (1ng/mL), IL-6 (10ng/mL), or in the presence of 50% serum shown to have concentrations of sCD23 in excess of 1000U/mL (Figure 9.7). Responses were only seen with TPA (4 ng/mL), or IL-2 (500 U/mL), and were maximal at 4 days. Responses to 4ng/mL TPA were highly variable, but in four cases responses were significantly enhanced by the presence of Interferon-alpha (Figure 9.8). Responses to 500U/mL IL-2 were also highly variable, but all responses were significantly reduced by the presence of Interferon-alpha (Figure 9.9).
Figure 9.7. Proliferative responses of CLL cells to (a) interferon-alpha (500U/mL), and (b) IL-1 (10U/mL).

E-rosette negative cells from 2 patients with CLL were incubated for various times with cytokines, and proliferation determined by measuring $^3$H-thymidine uptake over the last 16 hours of incubation.
Figure 9.7. Proliferative responses of CLL cells to (c) IL-2 (500 U/mL), and (d) IL-4 (1 ng/mL).
Figure 9.7 Proliferative responses of CLL cells to (e) IL-6 (10 ng/mL), and (f) TNF (500U/mL).
Figure 9.7 (g,h). Proliferative responses of CLL cells to (g) 50% serum, and (h) TPA (4 ng/mL).
Figure 9.8. Proliferative responses of CLL cells to TPA (4 ng/mL for 4 days).
Data are given for each of 10 patients studied.
Figure 9.9. Proliferative responses of CLL cells to IL-2 (500U/mL) for 4 days.
Apoptotic responses of CLL cells to cytokines in vitro

CLL cells were incubated for 18 hours in the presence or absence of a variety of cytokines, and with or without \(10^{-5}\)M hydrocortisone, and the amount of DNA fragmentation assessed. CLL cells incubated alone had a high level of spontaneous DNA fragmentation at 40.6±8.2\%, which was increased to 62.8±11.0\% in the presence of \(10^{-5}\)M hydrocortisone. Incubation in the presence of either interferon-alpha (500U/mL), Interferon-gamma (500U/mL), IL-1\(\beta\) (10U/mL), IL-4 (1ng/mL), IL-6 (10ng/mL), IL-2 (500U/mL), or in the presence of 50\% serum (shown to have over 1000U/mL of sCD23) was able to significantly reduce the level of both spontaneous and hydrocortisone induced DNA fragmentation (Figure 9.10). TNF-\(\alpha\) (500U/mL) did not significantly alter the level of DNA fragmentation.
Figure 9.10. Inhibition of both spontaneous and hydrocortisone induced apoptosis by cytokines. CLL cells were incubated for 18 hours with each condition, and DNA fragmentation determined using the diphenylamine reaction. Data are mean±SE of 3 experiments on separate patients.
DISCUSSION

The recognition that normal B-cell development is regulated by a variety of soluble growth and differentiation factors (Hirano & Kishimoto 1988, Kinkade et al 1989, Steel & Hutchins 1989, Callard 1990), has led to a great deal of interest in the role of these factors in the pathophysiology of B-cell lymphoproliferative disorders (Foa et al 1988, Foa et al 1991). Many candidate growth factors have been suggested, including IL-1, IL-2, IL-4, IL-6, interferon-alpha, interferon-gamma, TNF-alpha and sCD23. The involvement of cytokines in the autocrine behaviour of B-CLL remains controversial and revolves around several important questions, namely whether CLL cells express receptors for cytokines, whether CLL cells can produce cytokines or whether other cell types are producing them, and whether they can respond to cytokines. These questions are discussed below with particular reference to the potential growth factors studied.

Do CLL cells express cytokine receptors?

There is much evidence to suggest that CLL cells express a variety of cytokine receptors on their cell surface. Direct and indirect evidence has demonstrated the presence of receptors for interferon-alpha (Dadmarz et al 1987), IL2 (Murphy et al 1987, Perri & Kay 1987, Touw et al 1987) and IL4 (Bancherau 1991). Receptors for TNF-α are expressed in vitro, but some reports suggest that they may not be expressed in vivo (Digel et al 1990). Some authors have failed to demonstrate receptors on CLL cells for IL-6 (Hahn et al 1991), and interferon-gamma (Ucer et al 1986). It is not yet clear whether CLL cells express receptors for
IL-1, and the ligand for sCD23 is unknown. These cytokines may still exert an effect in spite of the inability of some studies to detect receptors, for example, receptors may be present at low levels. Alternatively, cytokines may exert their effects by stimulating the release of secondary cytokines from other cell types.

**Do CLL cells produce cytokines?**

CLL cells have been shown to produce both IL-1β (Pistoia et al 1986, Morabito et al 1987, Schena et al 1992), IL-6 (Biondi et al 1989, Hahnet al 1991, Schena et al 1992), TNF-α (Cordingley et al 1988, Foa et al 1990, Schena et al 1992), and sCD23 (Sarfati et al 1990, Fournier et al 1992). Presumably CLL cells are capable of producing interferon-alpha, as this is probably a property of all vertebrate cells (De Maeyer & De Maeyer-Guignard 1988), but this has not been conclusively demonstrated. CLL cells do not appear to be capable of expressing IL-2 (Schena et al 1992), IL4 (Schena et al 1992, Fournier et al 1992) or Interferon-gamma (Fournier et al 1992). Although the lack of production of these factors by CLL cells precludes an autocrine model of CLL involving these factors, their production by other cells such as activated T-cells and monocyte/macrophages raises the possibility of their involvement in paracrine control in the growth of CLL cells. There is evidence in CLL that T-cells and monocyte/macrophages may be activated either constitutively or by interferon-alpha therapy.

The results in this chapter demonstrate that some of the growth factors that are potential candidates for autocrine growth factors in CLL can be detected in the peripheral blood of patients.
It is clear that the levels of these growth factors are highly variable between patients. This may reflect the heterogeneity of the disease, or may be due to other factors. For example, levels of IL-1 receptor antagonist or soluble TNF receptor may influence both detection of and response to these cytokines. Furthermore, the levels of these growth factors in some patients show changes which reflect the response to interferon-alpha therapy. This may be due to the fact that CLL cells themselves produce the cytokines, and hence changes in serum levels of these growth factors directly reflect the number of CLL cells present. However, these factors can also be produced by other cells, particularly T-cells and macrophages (Hamblin 1988, Sarfati et al 1990). An alternative hypothesis is that interferon-alpha may directly regulate cytokine production by CLL cells. It has been demonstrated, for example, that interferon-alpha can reduce the levels of mRNA for TNF, IL-1 and IL-6 in CLL cells (Heslop et al 1990).

**Can CLL cells respond to cytokines?**

There is still much controversy surrounding the ability of CLL cells to respond to activation signals in a similar manner to normal B cells. Much of the controversy arises from the difficulty of identifying the normal counterpart of the CLL B cell, and the heterogeneity of normal B-cell populations. Most of the evidence suggests that CLL cells are much more difficult to induce to proliferate or differentiate in response to a variety of mitogenic signals than normal B-cells.

Many cytokines have been implicated as growth factors for CLL cells either alone or in combination, including IL-1 (Scala et
al 1986), IL-2 (Lantz et al 1985, Giovarelli et al 1988), IL-4 (Ghaderi et al 1988), IL-6 (Hahn et al 1991), TNF (Foa et al 1990, Hahn et al 1991, Digel et al 1989, Cordingley et al 1988), and even interferon-alpha (Ostlund et al 1986). However these data have been challenged on a number of grounds, including the pleiotropic nature of the cytokines employed, the need for co-stimulation by phorbol esters or mitogens, the difficulty of identifying the responding cells due to contamination by T-cells or B-cells even in highly purified cell systems, and the lack of specificity of $^3$H-thymidine uptake (Drexler 1988).

The results presented in this chapter add a further complication to the attempts to assess the role of cytokines as autocrine growth factors in CLL, and demand a more radical reassessment of much of the data. Incubation with TPA and IL-2 was able to induce $^3$H-thymidine uptake, but this may well depend upon the presence of contaminating T-cells. Exposure of CLL cells to a variety of cytokines, including IL-1, IL-4, IL-6, Interferon-alpha, Interferon-gamma, did not induce proliferation, but inhibited the level of apoptosis in these cells. Clearly if $^3$H-thymidine uptake is compared between cells in the absence of a particular cytokine, where 40% of the cells are dying or dead, and those in the presence of a cytokine in which most of the cells are still alive, false conclusions may be drawn. If $^3$H-thymidine uptake is used to assess proliferation, it may appear that the cells in the presence of cytokine are being induced to proliferate, when in fact there are simply more of them to respond. Similarly, small numbers of contaminating normal B-cell or T-cells may have a selective growth advantage under conditions where most of the neoplastic cells have apoptosed, and their
contribution to $^3$H-thymidine uptake will be exaggerated. This may confuse analysis of both growth and differentiation in vitro. Interestingly, TNF-α was not able to inhibit apoptosis in these experiments. Although, under the conditions employed, it was not possible to demonstrate increased $^3$H-thymidine uptake, many authors have suggested that TNF-α is able to induce proliferation. The observation that apoptosis is unaffected in the presence of TNF-α suggests that any proliferation seen must be due to a small subset of CLL cells or to residual normal B cells or contaminating T cells.

Whether these results reflect similar requirements of CLL cells for cytokines in vivo remains to be clarified. Most studies on cell kinetics in CLL indicate that CLL cells are long-lived in vivo and that cell turnover is low (Stryckmans et al 1977). This suggests that CLL cells are not undergoing apoptosis in large numbers in the circulation. However, the failure to demonstrate circulating cytokines in all patients with CLL, and the poor protection offered against apoptosis by autologous or allogeneic CLL serum, suggests that CLL cells may have other survival pathways in vivo. The possibility of designing therapeutic strategies to manipulate such survival pathways or to enhance apoptosis of CLL cells is intriguing.

These results suggest that CLL cells in vitro have promiscuous requirements for cytokines, not for proliferation or differentiation but for cell survival. Recently, a method for the measurement of apoptosis has been described which employs flow cytometry (Nicoletti et al 1991). Preliminary results using this technique compare very well with the diphenylamine method. If combined with immunophenotyping, it may be possible to look at
apoptosis, viability, phenotype, level of bcl-2 expression and cell cycle stage on the same cells. This will be a powerful tool in unravelling the mysteries of the growth factor requirements of CLL cells.
CHAPTER 10
GENERAL DISCUSSION, CONCLUSIONS
AND FUTURE DIRECTIONS

The results presented in this thesis demonstrate that interferon-alpha has a number of important biological effects on CLL cells. However, the essential pleiotropic nature of interferon-alpha makes it difficult to form a comprehensive picture of the ways in which the effects observed in vitro relate to those in vivo. Understanding of these processes would not only help to optimize the rational use of interferon-alpha as therapy, but would also contribute to the development of novel approaches to therapy based upon a clearer understanding of neoplasia and anti-neoplastic strategies. The population dynamics of the malignant cells in CLL are simplified and summarized in Figure 10.1, and the effects of interferon-alpha are discussed with reference to this model.

Haematological responses
Clearly interferon-alpha therapy does have an effect on the numbers of circulating lymphocytes in early stage CLL, but the responses are highly variable in extent, duration and kinetics. These observations prompt several important questions. Firstly, several reports have shown that interferon-alpha therapy is not effective, or only poorly so, in CLL patients with advanced disease. While this might reflect the evidence from experimental animals which suggested that small tumour burdens are more susceptible than large ones to the anti-neoplastic effects of interferon-alpha, it is important to consider the changes that
might occur during the evolution of CLL. The first possibility is that the CLL clone may acquire a more resistant or aggressive phenotype during the course of the disease, possibly by accumulating genetic mutations. These changes may be reflected in the clinical progression of the disease, which may eventually result in transformation. Secondly, the disease may change by the selection of sub-clones. Although the disease is well characterised as a clonal disorder, the population of CLL cells in any one patient is clearly heterogeneous with regard to morphology and expression of cell surface markers. Whether this reflects different stages of differentiation within the clone, or distinct sub-populations is unclear, but suggests ways in which the disease can progress to a more resistant form. Similarly, the heterogeneity of the disease may offer an explanation for the fact that only partial responses were seen to treatment in this and other studies. Presumably due to the nature of the disease all cells are equally exposed to interferon-alpha in the circulation. However it is not clear which cells within the population are resistant to interferon-alpha and which are susceptible. Nor is it understood whether the resistance occurs due to differential expression of receptors for interferon-alpha or whether it is due to intracellular mechanisms. Both have been shown to occur in experimental models. This poses the problem that prolonged interferon-alpha therapy may accelerate disease progression by selecting resistant clones. It is also possible that the differences in susceptibility reflect changes in the age or state of differentiation of the neoplastic population. This raises a further complication in that our knowledge of the natural history of the CLL cell is limited. In particular the identity and location
of the clonal parent is not known. It is clear that most if not all of the circulating CLL cells are in stage Go of the cell cycle, and it has proved difficult to demonstrate that CLL cells have enough proliferative activity to account for the accumulation of the malignant cells. It is possible that the proliferating cell or cells are in the lymph nodes and that it is their progeny which circulate. However, these cells have not been identified. If interferon-alpha is exerting its effects by inhibiting proliferation of these parent cells then it would be important to identify these cells, and their responses to interferon.

Whether interferon-alpha therapy can affect the stage of differentiation of the circulating CLL cell is difficult to determine. There was no evidence in this study of maturation to plasma cells, either by inspection of bone marrow or by monitoring changes in immunoglobulin levels. However, induction of differentiation could also lead to abortion, in a manner analogous to the mechanism of clonal abortion postulated to operate in B cell tolerance. In this model, absence of secondary signals neccessary for normal B cell development following stimulation by self antigen leads to the death of the clone. Furthermore, CLL cells have many features in common with the model of clonal anergy in B cell tolerance. CLL cells often express antibody against self determinants, but are functionally incompetent and do not normally secrete these antibodies into the serum. Also, CLL cells have not undergone the somatic hypermutation process, suggesting that they have been 'turned off' before conventional clonal selection processes take place. It is possible, therefore, that CLL cells represent a population of B cells which have been incompletely or inadequately tolerized, and
perhaps have later acquired genetic mutations which render them malignant.

**Immune effector mechanisms**

**T cells**

Although there was no evidence in this study that T cells were activated by interferon-alpha therapy, there was the intriguing observation that there is an increased proportion of T cells expressing serine esterase in untreated CLL patients. This suggests an increase in the number of cytotoxic T cells in these patients. This increase is particularly noticeable in the CD4 positive subset, in which serine esterase positivity is almost completely absent in normal individuals. The differential expression of serine esterase in these CD4 cells offers a unique opportunity to determine whether subsets of CD4 positive cells, distinguished by the cytokines they secrete, exist in man as has been shown in mouse cell lines. It might be expected that the serine esterase positive CD4 positive cells would secrete interferon-gamma, TNF-β and IL-2, corresponding to the TH1 subset in the mouse cell lines, and the serine esterase negative population might correspond to the TH2 subset secreting IL-4, IL-5 and IL-6. Furthermore it might be possible to grow these cells in culture in order to determine whether they represent true sub-populations or different maturational stages of a single lineage. An alternative explanation might be that the serine esterase positive cells represent an oligoclonal immune response to an antigen. The intriguing question is the nature of that antigen. It is possible that the response is a consequence of the immune paresis in CLL patients and represents a reaction to the increased
incidence of infection. This seems unlikely however in these patients with early stage disease. A second possibility is that this reflects an anti-tumour immune response in these patients. This is an exciting prospect in terms of the immunotherapy of malignant disease, but of course if it is a true anti-neoplastic response it has been ineffective. It is not clear why this response would not be effective but the activation of the TH1 subset and secretion of cytokines could of course lead to paracrine stimulation of the CLL cells themselves. A third possibility is that the T cells are reactive against an infectious agent, and this reflects the possibility of a viral aetiology for CLL. The virus may infect the B cells directly, or the B cell clone may reflect activity against heterologous infected cells. It is possible that due to self reactivity the B cell clone has been tolerized, as discussed above, but is maintained by excess cytokine secretion from the activated T cells. It is tempting to speculate that interferon-alpha therapy may exert its effect by interfering with the reproduction of the virus, and therefore abrogating the immune response and hence stimulation of the CLL clone. This conjecture raises the rather startling possiblity that immunosuppressive therapy may have a role in treatment of CLL.

**Monocytes/macrophages**

This study demonstrates that interferon-alpha therapy in CLL leads to an increase in circulating levels of M-CSF and neopterin. These results suggest that there is activation of monocytes/macrophages *in vivo*. How this activation leads to the haematological responses seen is not clear. It is possible that the reticuloendothelial system is directly involved by
removing the CLL cells by phagocytosis. There is much evidence suggesting that the CLL cell membrane is less flexible than that of normal B cells, eg absence of capping, changes in fatty acid composition of the lipid membrane, production of smear cells on the blood film. These properties of CLL cells could lead to increased clearance by the reticuloendothelial system, for example in the spleen, a process which would be enhanced by activation of the system. A second possibility is that interferon acts directly on monocytes/macrophages to induce cytokine production, which could lead to enhanced responses by neutrophils through the production of interferon-gamma, or T cells via a variety of cytokines. Alternatively, the changes in cytokine secretion may have effects on the CLL cells themselves via changes in the cytokine profile and/or in cytokine feedback loops. It is interesting to note that interferon-alpha therapy is most effective in hairy cell leukaemia, a disease characterised by monocytopaenia, and responses are accompanied by the restoration of monocyte counts. It is possible that this is due to increased M-CSF production induced by interferon-alpha, leading to increased monocytopoiesis, and cell activation.

**Adhesion and migration**

CLL is a disease characterised by the accumulation of lymphocytes in the peripheral blood rather than the lymph nodes, and it is this fact which is one of the most important criteria in distinguishing CLL from the non-Hodgkin's lymphomas. Our understanding of the physiological basis for lymphocyte adhesion and recirculation has advanced enormously since the phenomenon was first described by Gowans in the 1960's, but the relationship
of the physiological recirculation to the histological presentation of the non-Hodgkin's lymphomas and CLL remains unclear. However, even small changes in the distribution or recirculation kinetics in CLL could lead to marked differences in the number of cells in the peripheral blood (Figure 10.1). Furthermore, disease progression in CLL is associated with increased invasion of extravascular tissues by the malignant cells. These changes in distribution of the cells may also reflect the differential sensitivity of CLL to interferon-alpha therapy throughout the course of the disease. The observation that interferon-alpha increased the expression of L-selectin on CLL cells suggested that this might lead to increased migration to the peripheral lymph nodes. Although it was not possible to demonstrate increased binding to vascular endothelium in vitro it is possible that altered distribution is occurring in vivo. The dynamics of lymphocyte migration are not simply dependent on adhesion, and it is hoped to create new model systems to determine the factors involved in extravasation of lymphocytes, particularly in the lymph nodes. Once in the tissues, CLL cells could then come into close contact with macrophages activated by interferon therapy, and be removed by phagocytosis. It would be interesting to monitor lymphocyte distribution in patients before and after receiving interferon-alpha, focussing in particular on changes in the kinetics of recirculation of CLL cells. Furthermore, the HEV culture system established for this work will also prove useful in investigating the mechanisms involved in the pathophysiology of a number of chronic inflammatory conditions.
Figure 10.1 Model of population dynamics of CLL cells
Cell survival and cell death

Like many human tumour cells, CLL cells die within a short period of time when grown in culture. Addition of a variety of cytokines is able to support the survival of the cells, without promoting proliferation. This study demonstrates that CLL cells are highly promiscuous in terms of their requirements for these survival factors. How these requirements \textit{in vitro} reflect the situation \textit{in vivo} is not clear. It is possible that very low levels of a number of cytokines may synergize to maintain the CLL cells, or that possibly cell-cell interactions may promote their survival. Activation of the monocyte/macrophages by interferon-alpha may change the pattern of cytokines the cells are exposed to, and could therefore alter the natural history of the disease. Clearly, greater understanding of the ways in which the population is maintained will lead to possible therapeutic strategies.

The involvement of bcl-2 in the control of cell death is now well documented, and this study suggests it may play a role in the pathophysiology of CLL. The role of interferon-alpha in the regulation of bcl-2 expression in CLL was unexpected and counter-intuitive. In fact, the data suggest that interferon-alpha therapy could lead to disease progression by enhancing cell survival. However the apparent increase in expression both \textit{in vivo} and possibly \textit{in vitro} may reflect selection of cells expressing higher levels of bcl-2. Conversely, this may represent a response to interferon-alpha in order to counteract the anti-neoplastic effect. The fact that the CLL cells appear to have the components neccessary for programmed cell death already assembled, supports the notion that they may represent a tolerated population. However, the cells do manage to survive \textit{in}
vivo. The potential for the therapeutic exploitation is clear, and future work will attempt to dissect the cellular mechanisms involved in the balance between life and death of these cells (Figure 10.1).

**Synthesis**

The work presented here probably raises more questions than it answers. It seems likely that several cellular mechanisms are involved in mediating the effects of interferon-alpha in CLL. Increased extravasation of CLL cells due to increased expression of homing molecules could lead to increased contact with activated monocyte/macrophages. Reduction in the numbers of CLL cells could result from direct removal by phagocytosis by the activated reticulo-endothelial system, or by interferon-induced changes in the cytokine profile secreted by these cells. The changes in the cytokines available to CLL cells may alter the balance between cell survival and apoptosis.
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PUBLICATIONS AND SUBMISSIONS ARISING FROM WORK PRESENTED IN THIS THESIS


Jewell AP, Worman CP, Giles FJ, Goldstone AH, Lydyard PM Interferon-alpha therapy of chronic lymphocytic leukaemia leads to increased expression of bcl-2 and induces resistance to apoptosis (submitted for publication)

Jewell AP, Worman CP, Giles FJ, Goldstone AH, Lydyard PM Proliferative and apoptotic responses of chronic lymphocytic leukaemia cells to cytokines (submitted for publication)

Jewell AP, Worman CP, Giles FJ, Goldstone AH, Lydyard PM Levels of circulating cytokines in patients with chronic lymphocytic leukaemia on interferon-alpha therapy (in preparation)

Worman CP, Jewell AP, Giles FJ, Goldstone AH. T-cell activation in patients with chronic lymphocytic leukaemia on interferon-alpha therapy (in preparation)
PUBLICATIONS AND SUBMISSIONS ARISING FROM OTHER WORK DURING PERIOD OF STUDY


Jewell AP, van Schie R, Worman CP, Lydyard PM. Bispecific antibodies can mediate killing of chronic lymphocytic leukaemia cells by monocytes. (in preparation)