IMMUNE: PARAMETERS AS PREDICTORS OF RESPONSE TO MAINTENANCE THERAPY IN LOW GRADE NON-HODGKIN'S LYMPHOMA PATIENTS

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This dissertation is dedicated to the memory of my mother, Esmé Owen and to my father, Keith Owen and my children, Dominique and Bradley.

ABSTRACT

The non-Hodgkin's lymphomas (NHL) are a heterogeneous group of malignancies characterised by the uncontrolled proliferation of lymphoid cells. The Working Formulation divides these neoplasms into low, intermediate and high grade categories according to their natural history. Low grade NHL (LG-NHL) is clinically indolent whereas high grade NHL is more aggressive. Most LG-NHL patients respond well to chemotherapy, but are rarely cured, making death from LG-NHL virtually inevitable. In this study on LG-NHL patients, all patients received combination chemotherapy for 6 weeks, which consisted of cyclophosphamide, oncovin (vincristine) and prednisone (COP). After this treatment patients received either oral cyclophosphamide or alpha-interferon (α-IFN) as maintenance treatment for a period of two years. The ability to predict patients' treatment response at diagnosis would be extremely helpful for both the patient and the clinician. This would enable appropriate therapy to be instituted at diagnosis, whether it be cyclophosphamide or α-IFN, which might increase the subsequent survival of these patients, as this would prevent patients from receiving treatment to which they would not respond. Furthermore, patients who were unlikely to respond to either of these two treatments could be targeted for alternative treatment or form part of a clinical research trial. LG-NHL patients were assessed at diagnosis for a number of immune parameters. These included:- natural killer activity (NKA), α-IFN enhanced NKA, mitogen and antigen proliferation, lymphokine activated kill against both K562 and Daudi targets, phenotypic analysis of circulating lymphocytes, assessment of IL-2 levels after mitogen stimulation of lymphocytes, as well as the determination of plasma IL-2 levels. All these above-mentioned parameters were serially monitored over time in an attempt to predict early relapse. A statistically significant reduction in percentage of circulating CD3+ and CD8+ cells and an increase in percentage NK cells was found in patients at diagnosis as compared to normal controls. A reduction in percentage of circulating NK cells over time appeared to be a good prognostic indicator and an increase in percentage NK cells a poor prognostic indicator in this group of patients, although this was not statistically significant. When the *in vitro* α-IFN enhanced NKA was indicated as a percentage of NKA, a negative correlation appeared to exist between this *in vitro* response to α-IFN and the *in vivo* response, although this was not statistically significant (i.e. those patients showing the least α-IFN enhanced NKA were those that responded clinically and those with the highest α-IFN augmented NKA either relapsed or transformed to a higher grade of NHL). By incorporating the percentages of circulating lymphocytes present in the peripheral blood, into the multivariate discriminant analysis, it was possible to derive formulae to enable the prediction of response to either α-IFN or cyclophosphamide. This was a particularly exciting and apparently novel finding. The work presented here has therefore established both a possible negative indicator of α-IFN response (a high *in vitro* α-IFN response) and a positive indicator (the formula generated in the multivariate discriminant analysis using the flow cytometric phenotypic analysis). By

making using of both of these factors, it would increase the chances of patients being selected for appropriate forms of treatment and minimise the chances of patients suffering a relapse. The Kaplan-Meier curve indicated that cyclophosphamide treatment was more beneficial than α-IFN therapy in this group of patients, although this did not attain statistical significance. Verification of all of these above-mentioned findings would need further confirmation in a larger study.

DECLARATION

I hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. Parts of this dissertation have been presented at:- The John Humphrey course on Tumour Immunology, Romania, 1992; The 3rd Federation of African Immunological Societies Congress, Cape Town, 1997; The 26th Annual Meeting of the International Society for Experimental Hematology, Cannes, France, 1997.

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8th day of April, 1998.

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ADCC	-	Antibody dependent cellular cytotoxicity
AIDS	-	Acquired immune deficiency syndrome
ATCC	-	American Tissue Culture Collection
BRM	-	Biological Response Modifier
CD	-	Cluster of differentiation antigen
COP	-	Combination chemotherapy consisting of cyclophosphamide, oncovin (vincristine) and prednisone
cpm	-	Counts per minute
^{51}Cr	-	Sodium chromate 51
CTL	-	Cytotoxic T lymphocytes
ECOG	-	Eastern Co-operative Oncology Group
FasL	-	Fas ligand
FCS	-	Foetal calf serum
g	-	Gravitational attraction (9.81 metres/second^2)
GM-CSF	-	Granulocyte/macrophage colony stimulating factor
IFN	-	Interferon
Ig	-	Immunoglobulin
IL	-	Interleukin
IL-2R	-	IL-2 receptors
IU	-	International units
KAR	-	Killer cell activatory receptors
kDa	-	Kilodalton
KIR	-	Killer cell inhibitory receptors
LAK	-	Lymphokine activated killer
LGL	-	Large Granular Lymphocytes
LG-NHL	-	Low grade non-Hodgkin's lymphoma
µci	-	Microcurie
µg	-	Microgram
µl	-	Microlitre
M	-	Molar/molarity
MBq	-	MegaBecquerel
mg	-	Milligram
MHC	-	Major histocompatibility complex
MHC-NR	-	Major histocombatibility complex non-restricted
ml	-	Millilitre
mM	-	Millimolar/millimole
NHL	-	Non-Hodgkin's lymphoma

NK	-	Natural Killer
NKA	-	Natural Killer Activity
NKAR	-	Natural killer cell activatory receptors
NKIR	-	Natural killer cell inhibitory receptors
nm	-	Nanometre
PBMC	-	Peripheral blood mononuclear cells
PBS	-	Phosphate buffered saline
PHA	-	Phytohaemagglutinin
Postcop	-	After the completion of COP treatment
PPD	-	Purified protein derivative of *Mycobacterium tuberculosis*
RBC	-	Red blood cells
RPMI	-	Roswell Park Medical Institute media
SI	-	Stimulation index (mean cpm's in stimulated wells/mean cpm's in unstimulated wells)
SK-SD	-	Streptokinase-streptodornase
TCR	-	T cell antigen receptor
TGF-β	-	Transforming growth factor-beta
TIL	-	Tumour infiltrating lymphocytes
TNF	-	Tumour necrosis factor
U	-	Units

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1. INTRODUCTION

1.1.0. NON-HODGKIN'S LYMPHOMA

The non-Hodgkin's lymphomas (NHL) are a heterogeneous group of neoplasms, named after Thomas Hodgkins who, in 1832, first described patients with tumours of the "absorbent glands". Hodgkin's disease can be distinguished from NHL by the presence of Reed-Sternberg cells. Reed-Sternberg cells are considered to be the characteristic tumour cells of Hodgkin's disease. These cells are large with eosinophilic cytoplasm, striking nuclear morphology and a large eosinophilic inclusion-like nucleolus with a well-defined nuclear membrane and pale-staining chromatin. Classically these cells have two mirror-image nuclei. Also present are large cells of a similar appearance but with a single round nucleus and a very large nucleolus, designated mononuclear Hodgkin's cells (Jaffe *et al.*, 1993; Hancock, 1995; Sloane, 1987; Burrichter *et al.*, 1987; Bain *et al.*, 1996). The term lymphoma includes a number of neoplasias in which the malignant process usually involves the lymph nodes, spleen, and other lymphoid organs, but occasionally extralymphatic tissue is involved (Babior and Stossel, 1994). The term NHL includes more than a dozen neoplasms of the lymphoid system (Aisenberg, 1995). NHL's are characterised by the uncontrolled proliferation of lymphoid cells, predominantly B cells (Jaffe *et al.*, 1993; Hancock, 1995). Depending on the type and stage of evolution of NHL, malignant cells may be present in the peripheral blood, similar to the lymphocytic leukemic picture. There can be considerable overlap between lymphomas and leukemias (Babior and Stossel, 1994). All lymphomas are of monoclonal origin (Cotran *et al.*, 1989; Ozer *et al.*, 1983; Braylan *et al.*, 1975). Clonality of B-cells can be shown by the detection of either λ or κ light chain expression, rather than a mixture of both λ or κ light chains, which would be shown by normal B-cells. Malignant cells present in the haematological malignancies may not multiply at a rapid rate. Multiplication is often slower than in normal haemopoietic stem cells. The problem arises as a result of the failure of these cells to differentiate, as well as the immortality of these cells. These neoplastic cells suppress normal bone marrow cells and may invade normal tissue, impairing its function (Babior and Stossel, 1994; Smith, 1996). An oncogene that is associated with certain lymphomas is *Bcl-2*, which contributes to neoplastic expansion primarily by prolonging cell survival through its ability to inhibit genetically programmed cell-death (apoptosis). *Bcl-2* is an example therefore of an oncogene that does not affect cell proliferation, but causes an increase in cell survival (Babior and Stossel, 1994; Smith, 1996; Magrath, 1997). Oncogenes can be defined as genes that cause cell transformation via the production of transforming proteins (Widnell and Pfenninger, 1990). Apoptosis appears to be a mechanism for removing cells that are either:- redundant surplus cells; have completed their crucial yet temporary role; may be harmful to the organism (in this case lymphoma cells) (Osborne and Schwartz, 1995). See Figure 1 for a diagrammatic representation of the cell

cycle indicating the influence that the oncogene *Bcl-2* has on this cycle. It has been proposed that the absence of cell death rather than an increased proliferation of malignant cells may be responsible for the development of NHL, particularly low grade NHL (LG-NHL). *Bcl-2* blocks apoptosis and decreases the normal rate of cell death in follicular lymphomas (Shetty *et al.*, 1995).

NHL is diagnosed on the histological examination of a lymph node biopsy for effacement of normal architecture by a monotonous infiltrate of cells. The cell size is noted and the lymphoma is grouped into either a small or large cell class. When the majority of cells present are large with vacuolated nuclei and multiple large nucleoli, the lymphoma is classified as a large cell lymphoma. The arrangement of these cells is also noted, whether they have a tendency to form nodules, follicles, or a diffuse pattern throughout the node. The lymphoma is further described morphologically by how closely the cells present resemble normal lymphocytes. If the cells closely resemble lymphocytes, the lymphoma is described as being well-differentiated, as opposed to being poorly-differentiated, if the cells do not resemble lymphocytes. The nuclei of some lymphocytes have predominant infoldings and cleavages, and are thus further subdivided into cleaved or uncleaved (Babior and Stossel, 1994).

These neoplasms range from low grade, which is clinically indolent, to high grade, which is an aggressive form and unless treated, rapidly fatal (Jaffe *et al.*, 1993; Hancock, 1995). LG-NHL represents approximately 25% of all NHL patients (Kwak *et al.*, 1994). LG-NHL patients are able to survive for several years irrespective of the implementation of treatment, which serves merely to alleviate the unpleasant symptoms (Magrath, 1990). LG-NHL's have a high proportion of resting cells and a low proportion of cycling cells. This low proliferative rate of LG-NHL's account for the typically long median survival of these patients (Grogan, 1996).

Most LG-NHL patients respond well to chemotherapy, but patients are rarely cured (Magrath, 1990; Hancock, 1995). Chemotherapy can be defined as the treatment of malignant stem cells by the use of medications and hormones. Due to the fact that chemotherapy usually penetrates tissue well, it can be used in the treatment of patients with widely disseminated NHL (Babior and Stossel, 1994). LG-NHL's can transform from an indolent to a more aggressive form of NHL (Babior and Stossel, 1994). LG-NHL's generally become widely disseminated throughout the body and median survival time is 7-8 years (Magrath, 1990; Berkow and Fletcher, 1987; Hancock, 1995; Horning, 1994-a). Two thirds of LG-NHL patients survive for 5 years (Aisenberg, 1995). High grade lymphomas generally respond well to chemotherapy, with approximately one third of the patients being cured (Hancock, 1995).

Standard therapeutic regimens for LG-NHL have failed to cure these patients, and the median survival of LG-NHL patients has remained the same for the past 30 years. The reason for this unfavourable outcome in LG-NHL patients may be due to the fact that low grade lymphomas have a low mitotic rate and cytotoxic therapy is aimed primarily at rapidly proliferating cells. This low mitotic rate accounts for the relatively long natural history of this disease, but also explains why cytotoxic treatment is unable to cure these patients. It is clear that innovative treatment approaches are necessary. These methods could include:- agents which would stimulate apoptosis of lymphoid cells, the use of high dose chemotherapy to kill proliferating cells, and biologic therapies to eliminate minimal residual disease (Jaffe *et al.*, 1993; Horning, 1994-b).

1.1.1. FEATURES OF NHL

The features of NHL are summarised in Table 1.

TABLE 1. THE FEATURES OF NHL

Factor	Feature	Reference
Mortality	Account for 3-5% of deaths from malignant neoplasias.	Magrath,1990.
Morbidity	Incidence increased by 50% from 1973-1988. Overall incidence in UK = 8 cases per 100 000 per year.	Greiner *et al.*, 1995 Hancock,1995.
Age at onset	More common in elderly, but occurs in all age groups with a slight peak around adolescence. Low-grade 37% = 35-64 years; 16% <35 years. Rare in children.	Magrath, 1990; Berkow and Fletcher, 1987; Linch, 1990;
Male:female ratio	6:5	Hancock,1995.
Aetiology	Exposure to hair dye, herbicides, pesticides and fertilisers. Epidemiological evidence suggest that farm and meat workers are at greater risk from transmission of oncogenic viruses. Grain mill workers and motor vehicle and resin product manufacturing workers also appear at greater risk. Textile workers also seem to be at higher risk due to increased exposure to copper dust and ammonia. People employed in the agricultural, forestry and fishing industries and the construction and leather industries have a greater risk of developing NHL. At increased risk were plant farmers and gardeners, painters and plasterers, carpenters, brick and stone masons, plumbers, and roofers. Possible exposure to chlorophenols or phenoxy acids appeared to be the common agent in these occupations responsible for the higher risk of NHL. Increased risks were found among metal working, hair-care, painting and dry-cleaning occupations.	Greiner *et al.*, 1995; Carli *et al.*, 1994; Kwak *et al.*, 1993; Fritschi and Siemiatycki, 1996; Kwak *et al.*, 1994.
Clinical features	Low-grade - usually asymptomatic lymphadenopathy. May present with systemic symptoms.	Berkow and Fletcher, 1987; Jacobs and Bird,1983; Linch, 1990.
Treatment	Symptomatic for low-grade. Intensive chemotherapy for high-grade.	Hancock,1995.; Magrath, 1990; Ersboll *et al.*, 1985.
Diagnosis	Histological study of excised tissue.	Berkow and Fletcher, 1987.
Survival	Low-grade - median 7-8 years.	Hancock,1995.

1.1.2. CLASSIFICATION OF NHL

Much confusion and controversy exists over the classification of NHL. There are a number of different categorisation schemes, which results in difficulties when attempting to compare clinical therapeutic studies in which different systems have been employed (National Cancer Institute, 1982; Ersboll *et al.*, 1985; Nathwani, 1979; National Cancer Institute, 1985; Pugh,

1993). Further problems arise due to the fact that some NHL categories are easier to recognise and others more subjective (Harris *et al.*, 1994). Only the well-known and widely utilised classification schemes will be discussed here. In this study the Working Formulation was used, so a comparison will be made between that and other schemes to facilitate any correlation between trials.

The Rappaport Classification, proposed in 1956 and modified in 1966, has been the most commonly utilised in the United States (Table 2). This classification combines cytological appearance of cells with their growth pattern as either nodular aggregates or a diffuse infiltration throughout the node (Rappaport *et al.*, 1956; Byrne, 1977; Nathwani *et al.*, 1978; National Cancer Institute, 1985). This classification scheme was proposed prior to the knowledge of lymphocyte subsets, their activation and their specific anatomic location within the lymph nodes (Cotran *et al.*, 1989; Lukes and Collins, 1974-b).

TABLE 2. **MODIFIED RAPPAPORT CLASSIFICATION (1966)**

(Magrath, 1990; Berard, 1987; Ezdinli *et al.*, 1979).

Classification	Working Formulation related category	Description of terms
Nodular		Tumour cells clumped into recognisable nodules throughout the lymph node. Better prognosis than diffuse.
Lymphocytic, well differentiated	A	Well differentiated - Resembled normal lymphocytes in size and morphology. More differentiated the cell type, the greater the survival advantage than patients with large immature cell types.
Lymphocytic, poorly differentiated	B	Poorly differentiated - Lymphocyte-like cells, but containing irregular and angulated nuclei.
Mixed, lymphocytic and histiocytic	C	Mixture of lymphocytes and between 30 - 50% large tumour cells.
Histiocytic	D	Composed of greater than 50% large tumour cells containing abundant cytoplasm.
Diffuse		No follicles present in lymph node. Proliferating cells diffusely infiltrate or efface the architecture of the node.
Lymphocytic, well differentiated without plasmocytoid features	A	Well differentiated - Resembled normal lymphocytes in size and morphology. More differentiated the cell type, the greater the survival advantage than patients with large immature cell types.
Lymphocytic, well differentiated with plasmacytoid features	A	
Lymphocytic, poorly differentiated	E	Poorly differentiated - Lymphocyte-like cells, but containing irregular and angulated nuclei.
Lymphoblastic, convoluted	I	
Lymphoblastic, non-convoluted	I	
Mixed, lymphocytic and histiocytic	F	Mixture of lymphocytes and between 30 - 50% large tumour cells.
Histiocytic without sclerosis	G	
Histiocytic with sclerosis	G	
Burkitt's tumour	J	
Undifferentiated	J	
Malignant lymphoma, unclassified		
Composite lymphoma		

See Table 5 for description of Working Formulation and related categories.

In 1973, the Lukes-Collins classification was proposed and modified in both 1974 and 1976 (Table 3) (Lukes and Collins, 1974-a; Lukes and Collins, 1974-b; Lennert *et al.*, 1975; Nathwani *et al.*, 1978; Lennert *et al.*, 1983). This scheme attempted to overcome the shortcomings of the Rappaport classification (Cotran *et al.*, 1989). Lukes and Collins categorised the NHL's according to their cell of origin into tumours of three different cell types:- (1) T cells, (2) B cells, or (3) histiocytes (Cotran *et al.*, 1989; Lukes and Collins, 1974-a; Lukes and Collins, 1974-b; Lukes, 1971; Jaffe *et al.*, 1993). A major disadvantage of this classification system is the lack of correlation with prognosis. The cumbersome terminology of this classification scheme is also a limitation (Dorfman, 1977).

TABLE 3. **LUKES AND COLLINS CLASSIFICATION (1974)**

(Magrath, 1990).

Lukes - Collins classification	Working Formulation related category
Undefined cell type	
T-cell, type, small lymphocytic	A
T-cell, type, Sezary-mycosis fungoides (cerebriform)	
T-cell type, convoluted lymphocytic	I
T-cell type, immunoblastic sarcoma (T-cell)	H
B-cell type, small lymphocytic	A
B-cell type, plasmacytoid lymphocytic	A
Follicular centre cell, small cleaved	B,E
Follicular centre cell, large cleaved	D,G
Follicular centre cell, small non-cleaved	J
Follicular centre cell, large non-cleaved	D,G
Immunoblastic sarcoma (B-cell)	H
Subtypes of follicular centre cell lymphomas	
1. Follicular	
2. Follicular and diffuse	
3. Diffuse	
4. Sclerotic with follicles	
5. Sclerotic without follicles	
Histiocytic	
Malignant lymphoma, unclassified	

See Table 5 for description of Working Formulation and related categories.

A European group in 1974 proposed the "Kiel classification", due to the fact that the concepts were based chiefly on propositions by Lennert and co-workers from the University of Kiel, West Germany (Gerard-Marchant *et al.*, 1974; Lennert *et al.*, 1975; Nathwani, 1979; Lennert *et al.*, 1983). This categorisation has undergone several modifications both in 1975 and again in 1978 (Table 4). The Kiel classification is widely accepted in many European countries, which poses a dilemma in the comparison of therapeutic results across the Atlantic (Ersboll *et al.*, 1985; Lennert *et al.*, 1975; Nathwani, 1979). NHL's are divided into either low or high grade malignancy. Low grade malignancies end with the suffix "-cytic or -cytoid" and high grade tumours, the suffix "-blastic".(Gerard-Marchant *et al.*, 1974; Lennert *et al.*, 1975; Ersboll *et al.*, 1985). In the Kiel classification, criteria are presented which are both confusing and contradictory and the majority of pathologists find great difficulty in adopting this scheme for regular diagnostic purposes (Byrne, 1977).

TABLE 4. **KIEL CLASSIFICATION (1974)**

(Magrath, 1990).

Kiel classification	Working Formulation related category
Low-grade malignancy (ends with suffix "-cytic or -cytoid")	
Lymphocytic, chronic lymphocytic/ leukaemia	A
Lymphocytic, other	A
Lymphoplasmocytoid	A
Centrocytic	E
Centroblastic-centrocytic, follicular without sclerosis	B,C,D
Centroblastic-centrocytic, follicular with sclerosis	
Centroblastic-centrocytic, follicular and diffuse, without sclerosis	
Centroblastic-centrocytic, follicular and diffuse, with sclerosis	
Centroblastic-centrocytic, diffuse	F
Low-grade malignant lymphoma, unclassified	
High-grade malignancy (ends with suffix "-blastic")	
Centroblastic	G
Lymphoblastic, Burkitt's type	J
Lymphoblastic, convoluted cell type	I
Lymphoblastic, other (unclassified)	
Immunoblastic	H
High-grade malignant lymphoma, unclassified	
Malignant lymphoma, unclassified (unable to specify 'high grade' or 'low grade'	
Composite lymphoma	

See Table 5 for description of Working Formulation and related categories.

A further classification system, the Working Formulation, was devised with a strong clinical bias (Cotran *et al.*, 1989; National Cancer Institute, 1982; National Cancer Institute, 1985). This scheme was intended to merely clarify the confusion and not as a new classification. The Working Formulation is however widely used as a classification scheme mostly in America. This poses a problem in comparing different clinical trials, as the European pathologists favour the Kiel classification. By using the Working Formulation, pathologists and clinicians world-wide may continue using terminology from any classification and by using this formulation, it is possible to translate terminology from one scheme to another, thereby assisting in comparisons of clinical therapeutic trials (National Cancer Institute, 1982; National Cancer Institute, 1985; Pugh, 1993). The Working Formulation is entirely a morphological classification, based on clinicopathologic correlative studies and without any immunological methods being employed (National Cancer Institute, 1982; Berard *et al.*, 1987). NHL's are separated into three different groups; low, intermediate and high grade, which correlate with natural history and prognosis and aids in clinical decision making

(Magrath, 1990; Cotran *et al.*, 1989; Berkow and Fletcher, 1987; Smith, 1996). Each of these above mentioned prognostic categories contain various morphological groups, making use of a combination of the Rappaport and Lukes-Collins terminology (Cotran *et al.*, 1989; Ersboll *et al.*, 1985; Sommers and Rosen, 1983). The Working Formulation, however, does not take into consideration immunologic data and this has short-comings when it is used as an alternative classification. The Working Formulation does not distinguish between T and B lymphoid systems, or the stages of differentiation within each system. This results in the grouping together of biologically different entities and the separation of closely related ones (Pugh, 1993). See Table 5 for description of Working Formulation and related categories.

Even more recently, in 1994, a Revised European-American Classification (REAL classification) was proposed (Table 5). The classification system was given this name, as it seeks to revise and co-ordinate the currently used European and American lymphoma classifications. The REAL classification builds on the Kiel classification, simplifying some aspects and including some lymphomas that were omitted in the Kiel classification (e.g. MALT lymphomas). It was felt that the understanding of lymphomas, as well as the techniques available immunologically and in molecular biology over the past 15 to 20 years, has made it necessary to revise the classification scheme, as a purely morphological basis is no longer sufficient. In this scheme, the lymphomas are divided into three major categories: B-cell neoplasms, T-cell and postulated natural killer neoplasms, and Hodgkin's disease. The NHL's are also divided according to prognosis depending on the clinical behaviour of a tumour. The use of immunophenotyping and special staining techniques as well as molecular genetic studies are also included (Harris *et al.*, 1994; Chan *et al.*, 1994; Bain *et al.*, 1996; Pittaluga *et al.*, 1996). An advantage of this scheme is that it employs commonly used terminology with minimal introduction of new terms. This scheme has drawbacks in that many of the methods employed are not available at all medical centres. This classification scheme appears to be unnecessarily complex and does not correlate clinically as is the case with other classification systems. The REAL classification does not define prognostic groups. It also appears to be more scientifically correct than clinically useful and needs to be formally tested for reproducibility (Meijer *et al.*, 1995; Aisenberg, 1995; Ioachim, 1996; Mason and Gatter, 1995; Rosenberg, 1994; Bonadonna, 1995).

Table 5 compares the REAL classification and the Working Formulation, as well as describing and giving the relevant category of the Working Formulation. This aids in the comparison of the Working Formulation with all the other classification schemes previously described.

TABLE 5. **COMPARISON OF WORKING FORMULATION AND REAL CLASSIFICATION**

(Hiddemann and Unterhalt,1994).

Working Formulation	REAL Classification
Low-grade lymphomas	
Small lymphocytic (A)	Lymphocytic
	Lymphoplasmocytoid
	Marginal zone
Follicular small cleaved (B)	Follicle centre, follicular
Follicular mixed (C)	(small and mixed)
Intermediate-grade lymphomas	
Follicular large (D)	Follicle centre, large
Diffuse small cleaved (E)	Mantle cell
Diffuse mixed (F)	Follicle centre, diffuse (small)
Diffuse large cell (G)	
High-grade lymphomas	
Immunoblastic, large cell (H)	Diffuse large B cell
Lymphoblastic, convoluted and non-convoluted (I)	B-precursor large B cell lymphoma/leukemia
Lymphoblastic, small-non-cleaved (J)	

The Ann Arbor Staging System, which was originally described for Hodgkin's disease, is also used in NHL (Table 6). The biologic characteristics of Hodgkin's disease and NHL, however, differ as NHL does not usually follow an orderly progression via adjacent nodal groups and is usually at an advanced stage at diagnosis. The Ann Arbor Staging System is therefore found to have little prognostic value in NHL, although it continues to be used in staging NHL patients (Linch, 1990; Jacobs and Bird, 1983; Rosenberg *et al.*, 1971; Smith, 1996; Denham *et al.*, 1996).

TABLE 6. **THE ANN ARBOR STAGING**

(Magrath, 1990).

Stage I	Involvement of a single extranodal organ or site (1E)
Stage II	Involvement of two or more lymph node regions on the same side of the diaphragm, or localised involvement of an extranodal site or organ (2E) and of one or more lymph node regions on the same side of the diaphragm
Stage III	Involvement of lymph node regions on both sides of the diaphragm, which may also be accompanied by localised involvement of an extranodal organ or site (3E) or spleen (3S) or both. (3SE)
Stage IV	Diffuse or disseminated involvement of one or more distant extranodal organs with or without associated lymph node involvement
Category A	Asymptomatic
Category B	Symptomatic (Fever > 38°C, night sweats, and/or weight loss > 10% of body weight in the six months preceding admission are defined as systemic symptoms)

The majority of LG-NHL are at stage III or IV when detected, with only 15 to 20% being diagnosed at stages I and II (Berkow and Fletcher, 1987; Hiddemann and Unterhalt, 1994; Vose *et al.*, 1991). By the time sufficient neoplastic cells have accumulated in a patient to produce symptoms, more than 90% of NHL's are already disseminated (Magrath, 1990).

A major problem in any study involving LG-NHL patients, is that years of follow-up are necessary before any valuable results can be shown, due to the long natural history of this disease (Witzig *et al.*, 1995).

1.1.3. CLINICAL FEATURES/ASPECTS OF NHL

The incidence of NHL has increased by 150% in the USA during 1950-1985. Incidence rates have increased in all age groups (except the very young), all race groups, over all geographic areas of the USA, and in both sexes. A particular observation has been the marked increase in older age groups. The incidence of NHL continues to rise world-wide. This increase is independent of the acquired immune deficiency syndrome (AIDS) epidemic. Reasons for this higher incidence include the fact that NHL was often misdiagnosed as another malignancy, as well as the fact that some NHL were not previously classified correctly. These factors can still not account for such a dramatic increase in the incidence of

this disease. Although the rise in the incidence of NHL is apparent over all ages, the most striking increases are among the older age groups. As the greatest increase occurs in the elderly, this may disprove the theory that some occupational groups are at an increased risk of developing NHL, as the effect should surely be apparent in younger people. An increased exposure to herbicides has been suggested as a cause for this escalation in developed countries. Exposure to hair dye, herbicides, pesticides and fertilisers has also been implicated. Epidemiological evidence suggest that farm and meat workers are at greater risk from transmission of oncogenic viruses. Grain mill workers and motor vehicle and resin product manufacturing workers also appear at greater risk, as too are textile workers, who seem to be at higher risk due to increased exposure to copper dust and ammonia. People employed in the agricultural, forestry and fishing industries and the construction and leather industries have an increased risk of developing NHL. Plant farmers and gardeners, painters and plasterers, carpenters, brick and stone masons, plumbers, and roofers are at increased risk of NHL which is possibly due to exposure to chlorophenols or phenoxy acids which appears to be the common agent in these occupations. Increased risks were also found among metal working, hair-care, painting and dry-cleaning occupations. A recent article published by McMichael and Giles (1996) suggests that the increase in the incidence of NHL may be attributed to exposure to ultraviolet radiation which causes systemic immune suppression. The incidence of NHL appears to be affected more by systemic immune suppression than that of any other malignancy. Autoimmunity has also been recognised as a factor in lymphomagenesis Viruses also play an etiological role in lymphomagenesis, especially Epstein-Barr virus and human T-cell leukaemia virus-1. Hepatitis C virus has also been implicated in lymphoma. It appears therefore, that overstimulation as well as suppression of the immune system may predispose patients to lymphoma (Chappuis and Sappino, 1995; Aisenberg, 1995; Greiner *et al.*, 1995; McMichael and Giles, 1996; Hjalgrim *et al.*, 1996; Carli *et al.*, 1994; Kwak *et al.*, 1993; Fritschi and Siemiatycki, 1996; Kwak *et al.*, 1994; Kwak and Longo, 1996; Gale *et al.*, 1994).

LG-NHL's are usually first recognised by painless enlargement of one or more peripheral lymph nodes and progress slowly. These lymphomas may cause few problems for the patient initially. The enlargement of internal lymph nodes may cause symptoms by encroaching on internal organs. As a result of the generalised distribution of lymphoid tissue throughout the body, practically any organ can become affected should NHL arise in or near it. Bone marrow infiltration is frequent in lymphoproliferative disorders. When the bone marrow is extensively involved, the haemoglobin concentration and platelet and neutrophil counts may be reduced. In NHL's of B-cell lineage, bone marrow infiltration is more common in LG-NHL's than in high grade NHL's. Bone marrow infiltration is observed more often in B-cell lymphomas than in T-cell lymphomas. The clinical importance of bone marrow involvement is dependent on the category of NHL. Usually the prognosis of LG-NHL's patients with bone

marrow involvement remains unaffected (Babior and Stossel, 1994; Bain *et al.*, 1996; Grogan, 1996).

Prognostic factors in LG-NHL (summarised in Table 7) as described by some authors, include: tumour burden - determined by factors such as Ann Arbor stage, bone marrow involvement, $\beta_2$microglobulin and number of extranodal sites; host factors - including age, sex, systemic symptoms, haemoglobin; presence of B symptoms (fever, night sweats, unexplained weight loss >10%) and response to treatment. Based on these factors, prognostic indices have been formulated (Horning, 1994-a; Shipp *et al.*, 1993; Kwak *et al.*, 1993; Coiffier *et al.*, 1993; Pasquini *et al.*, 1994). The International Prognostic Index considers age, performance status (according to the Eastern Cooperative Oncology Group as discussed in Section 1.1.5.1.), stage, extranodal involvement and elevated serum lactate dehydrogenase, but does not include tumour cell proliferation rate (Joensuu *et al.*, 1994; Hermans *et al.*, 1995). Witzig and co-workers (1995), recommend that a measure of tumour cells in the S-phase of the cell cycle be included in this prognostic index (see Figure 1 for a diagrammatic representation of cell cycle). The S-phase of the cell cycle is the DNA synthesis phase, where DNA content doubles in preparation for cell division. The proliferative rate of NHL can be measured by estimating the proportion of cells in the DNA synthesis phase. DNA binding dyes, such as propidium iodide are used, and DNA content histograms generated by flow cytometry. The percentage of S-phase fraction can then be determined using computer software. Alternatively, cell proliferation can also be determined by staining for the nuclear antigen Ki-67, which is a monoclonal antibody that marks a nuclear protein present in cycling cells. The prognostic value of the S-phase fraction varies in different studies, but it has been shown that LG-NHL patients demonstrating tumours with less than or equal to 5% Ki-67 positive cells, had a statistically significant better chance of survival (Witzig *et al.*, 1995; Miller TP *et al.*, 1994; Armitage, 1993). The mean S-phase fraction for the different grades of NHL, according to the Working Formulation, were determined in a study by Joensuu and co-workers (1994). The mean S-phase fraction for LG-NHL was found to be 4.8%, for Intermediate grade NHL, 10.3% and for High grade NHL, 11%. These groups could be further divided prognostically into subgroups by the use of these S-phase fractions and the comparison to the median for each group i.e. Low grade, Intermediate grade, or High grade.

Parker and co-workers (1994), found that LG-NHL patients with lymphocyte counts below 1 x 10^9/l prior to the commencement of treatment, demonstrated worse survival than patients with higher lymphocyte counts. The majority of patients presented with lymphocyte counts between 1-3 x 10^9/l, however patients with lymphocyte counts of greater than 3 x 10^9/l showed similar survival. In this same study, it was shown that serum immunoglobulin (Ig) levels correlated with survival. Using a cut-off point of 10g/l for IgG, it was found that

patients with < 10g/l showed significantly worse survival than patients with IgG of > 10g/l. It was speculated that this finding was as a result of disease progression rather than a pre-existing immunodeficiency. This is possibly due to the fact that the malignant B cells do not produce immunoglobulin and as their numbers increase relative to normal B cells this causes a decrease in the serum immunoglobulin.

Early detection of relapse following a response in NHL patients is important as this might increase the subsequent survival of the patient. In a study conducted by Weeks and colleagues (1991), only 6% of relapses were detected before the appearance of overt symptoms. This was despite diligent serial follow-up measures which included radiographic studies and gallium scans. In this same study only serum lactate dehydrogenase successfully detected preclinical relapse with a sensitivity of 42% and a specificity of 85% (Kwak *et al.*, 1993). Clearly a more sensitive method for detection of relapse is necessary.

TABLE 7. **PROGNOSTIC FACTORS IN LG-NHL AT DIAGNOSIS**
(Horning, 1993).

Clinical characteristic	Prognosis
Limited Ann Arbor stage	favourable
Age (> 60-70 years)	unfavourable
Male gender	unfavourable
Systemic symptoms	unfavourable
Good performance status (according to the Eastern Cooperative Oncology Group criteria as discussed in section 1.1.5.1.)	favourable
≥ 2 extranodal sites	unfavourable
Bulky tumour	unfavourable
Hepatosplenomegaly	unfavourable
> 20% bone marrow involvement	unfavourable
Normal hemaglobin	favourable
Elevated LDH	unfavourable
β_2 microglobulin >3	unfavourable

(LDH = lactic dehydrogenase)

1.1.4. PATHOPHYSIOLOGY OF LOW-GRADE B-CELL NHL

Malignant B cells display a lack of differentiation as a result of genotypic transformation, probably caused as a result of structural chromosome abnormalities affecting genes involved in cell cycle control (such as *Bcl-2*). Mutations have been demonstrated in lymphomas which could deregulate B-cell development. Cytokines do not appear to play a primary role in lymphomagenesis, however they may provide stimuli for malignant clone proliferation. *In vitro* studies have found secretion of cytokines such as tumour necrosis factor (TNF) alpha,

interleukin (IL) 6 and B-cell growth factor (IL-4) by malignant B cells. T-cell cytokines such as IL-2, IL-4 and IL-10 enhanced the proliferation *in vitro* (Schuler *et al.*, 1995). IL-6 appears to be a growth factor *in vitro* for some lymphomas (Kwak *et al.*, 1994). Low-grade malignant B cells are difficult to culture *in vitro* thus highlighting the necessity of growth stimulation provided by cytokines and direct cell-cell interactions with either T lymphocytes or other cells. Malignant B cells accumulate in lymphoid and extralymphatic tissue, which could be as a result of an inhibition of programmed cell death (apoptosis). See Figure 1 for a diagrammatic representation of the cell cycle indicating the influence that the oncogene *Bcl-2* has on this cycle. *In vitro* inhibition of malignant B cell apoptosis has been described for IL-4, interferon (IFN) gamma (γ) and IFN alpha (α) (Schuler *et al.*, 1995).

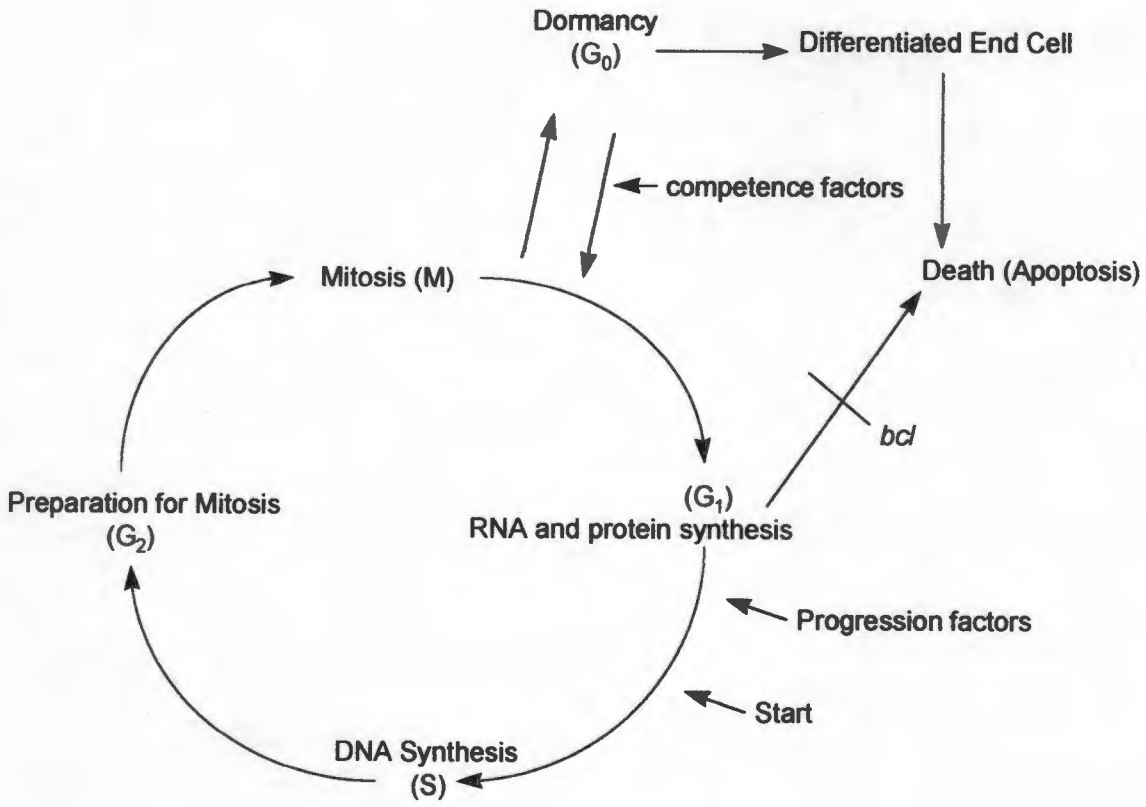


FIGURE 1. A diagrammatic representation of the cell cycle.
The cell cycle can be divided into four phases: G_1 (G = gap), S, G_2 and M.
G_1 phase. Immediately after mitosis, cells enter the G_1 phase. The cell synthesises and accumulates RNA, enzymes and proteins necessary for DNA synthesis. Cells either progress irreversibly into DNA synthesis (S phase) or become resting cells and enter G_0 phase. These cells may either remain in G_0, return to the cell cycle, or become differentiated end cells, which are incapable of cell division. The proposed role of the oncogene, *bcl*, is clearly indicated.
S phase. The S phase starts as soon as DNA synthesis begins. DNA content doubles in preparation for cell division. Thereafter cells enter the G_2 phase.
G_2 phase. This is the pre-mitotic phase. DNA synthesis ceases, but specialised protein and RNA synthesis continues. Microtubular precursors of the mitotic spindle are synthesised. Cells shift from G_2 to M phase.
M phase. This is the mitotic phase. Protein and RNA synthesis cease abruptly. Genetic material is segregated in an orderly manner. Cell division occurs and the two daughter cells enter either G_1 or G_0 phase (Babior and Stossel, 1994; Widnell and Pfenniger, 1990).

1.1.5.0. TREATMENT OF NHL PATIENTS

Treatment of NHL has changed very little over the past decades (Ersboll *et al.*, 1985; Hiddemann and Unterhalt, 1994). As mentioned previously, LG-NHL patients are able to survive for several years whether or not they undergo treatment. Treatment serves merely to alleviate the unpleasant symptoms of NHL (Macgrath, 1990). The treatment of choice for NHL patients diagnosed at stage I and II is irradiation, although, as mentioned previously, patients are rarely diagnosed at these stages (Hiddemann and Unterhalt, 1994). Treatment of stage III and IV LG-NHL patients remains controversial, with a "watch and wait" policy being recommended by some since aggressive combination chemotherapy has not been shown to significantly impact the overall survival of patients (Flecknoe-Brown, 1995; Hiddemann and Unterhalt, 1994; Vose *et al.*, 1991). LG-NHL are thus incurable using standard chemotherapy regimens, because although most NHL patients respond well to current chemotherapy this response is not sustained (Schuler *et al.*, 1995). Attaining a response in these patients with chemotherapy is not a major clinical hurdle; rather, maintaining the remission is the major clinical challenge as relapse is fairly common and treatment is not curative and does not affect median survival. The continuous relapse pattern makes death from LG-NHL virtually inevitable (Macgrath, 1990; Parkinson *et al.*, 1993; Rohatiner, 1991; Gaynor and Fisher, 1991). The goal when treating LG-NHL patients, is to selectively destroy the tumour stem cells with minimal damage to healthy stem cells. Surgery is usually not a viable option in the treatment of LG-NHL patients, due to the fact that tumour cells are usually disseminated at diagnosis (Babior and Stossel, 1994). Almost all LG-NHL patients have relapses at a rate of about 15% per year (McLaughlin *et al.*, 1993). It is clear that innovative treatment approaches are necessary in the treatment of LG-NHL patients. These methods could include agents which would stimulate apoptosis, in conjunction with the use of high dose chemotherapy to kill proliferating cells and biologic therapies to eliminate minimal residual disease (Jaffe *et al.*, 1993).

1.1.5.1. TOXICITY AND RESPONSE TO TREATMENT

The Eastern Cooperative Oncology Group (ECOG) have presented criteria for the standardisation of toxicity and response to treatment. ECOG stresses that such a standardisation is important for researchers conducting clinical trials. Extremely detailed criteria have been proposed by this group, but a brief summary follows. A scale to evaluate **toxicity** from 0-5 has been formulated as:

0 = none

1 = mild

2 = moderate

3 = severe

4 = life-threatening

5 = lethal.

The ECOG **performance status** ranges from 0-5 as follows:

0 = Fully active.

1 = Restricted in physical activity. Able to carry out light work.

2 = Ambulatory and capable of self care. Unable to work at all. Up and about 50% of the day.

3 = Capable of limited self care. Confined to bed or chair for more than 50% of the day.

4 = Completely disabled. Incapable of self care. Confined totally to bed or chair.

5 = Dead.

Response criteria are defined by ECOG as:

1. Complete response - disappearance of all clinically detectable malignant disease for at least 4 weeks. Lymphoma patients who at diagnosis presented with abnormal lymphangiograms and who have stable and marked decrease in size of lymphangiographically abnormal nodes are considered complete responders if all other measurable, evaluable, and pathologically demonstrable disease is in complete response.
2. Partial response - greater than or equal to 50% reduction in tumour area for at least 4 weeks, as well as no increase of >25% in the size of any malignant area. No new appearance of malignancy.
3. Stable disease - no significant change in measurable or evaluable disease for at least 4 weeks.
4. Progressive disease - significant increase in the size of lesions that were present at the start of therapy or after a response. Appearance of new malignant sites. Stable disease, but combined with a decline in ECOG performance status of greater than or equal to 1 level.

5. No evidence of disease - lack of clinically determined malignant disease in nonmeasurable, nonevaluable patients.
6. Onset of response - time between initiation of treatment and the onset of either partial response or complete response.
7. Duration of response - time from onset of partial response (even if patient subsequently has a complete response) until clear evidence of progressive disease.
8. Overall survival is calculated from the first day of treatment to death (whatever the cause) (Coiffier *et al.*, 1993; Oken *et al.*, 1982).

The appearance of second neoplasms after treatment for NHL has occasionally been reported. Patients undertaking therapy may therefore be at an increased risk of developing second neoplasms (Kwak *et al.*, 1993).

1.1.5.2. CHEMO-IMMUNOTHERAPY

For chemotherapy to be successful, it must eradicate all tumour cells. It is usually not possible to eradicate all tumour cells, as some cells are resistant to the drug, and there are also the constraints of a maximum tolerated dose. Immunotherapy is the stimulation of the hosts own anti-tumour immunity by means of treatment. The term "biological response modifier" (BRM) is often used to refer to these approaches to immunotherapy. Therapy could include IFN, which stimulates the host's own defence mechanism and is considered a BRM (Foon, 1989). BRM's are agents and approaches whereby the mechanism of action involves the patient's own biological response. BRM's act by:- (1) increasing the host's defenses by administering natural biologicals (or synthetic equivalents) as effectors or mediators of an antitumour response, or (2) increasing the individual's own antitumour responses, through either enhancement or restoration of effector mechanisms, or by decreasing the unfavourable component of the host's reaction, or both, or (3) enhancement of the patient's responses to modified tumour cells or vaccines which may stimulate a better response for example by making the tumour cells more susceptible to killing (Geerligs *et al.*, 1993). Toxicities usually differ between chemotherapy agents and immunostimulating agents, therefore a combination should increase the therapeutic index. There are differences between the response to chemotherapy and immunotherapy: (1) Unless patients demonstrate a complete response, while using chemotherapy, they do not experience prolonged survival. Patients exhibiting even a partial response, while using immunotherapy, usually experience prolonged survival; (2) The effect of chemotherapy will only last as long as the treatment, whereas with immunotherapy, it may continue for weeks or even months after completion of treatment; (3) The response to chemotherapy is usually directly dose-related, but this is not as marked with immunotherapy, and (4) chemotherapy appears more

effective for bulky disease, whereas immunotherapy is probably more effective for minimal residual disease (Kedar and Klein, 1992).

Alpha Interferon (α-IFN) and cytotoxic drugs e.g. cyclophosphamide and vinca alkaloids appear to have a synergistic or additive effect when administered to LG-NHL patients (Solal-Celigny *et al.,* 1993; Rohatiner, 1991).

The aim, in a clinical trial would therefore b e to reduce the tumour load using chemotherapy, prior to the use of immunotherapy (Kedar and Klein, 1992).

1.1.6.0. ALPHA INTERFERON

Interferons are a group of naturally occurring proteins and glycoproteins, first described by Isaacs and Lindenmann in 1957, that have large molecular weights and demonstrate a number of biological activities, including antiviral, antitumour activity and regulation of the immune system. Several types of IFN exist that differ in amino acid composition and carbohydrate content. For several years IFN was regarded only as a mediator of viral interference (hence the name, from "to interfere") (Geerligs *et al.*, 1993; Crowther, 1991). IFN's are secreted by cells following exposure to a variety of biologic and chemical stimuli (Louie *et al.*, 1981; McLaughlin, 1993). IFN's have been classified as α-interferon, β-interferon and γ-interferon. α-IFN's are produced by leukocytes, γ-IFN by T lymphocytes and β-IFN by fibroblasts and epithelial cells (Crowther, 1991; Gilewski and Richards, 1990). IFN's are also responsible for growth and differentiation of normal B lymphocytes and demonstrate a direct antiproliferative effect against tumour cells and stimulatory effects on antitumour cells. IFN's also cause induction or augmentation of expression of membrane antigens on tumour cells which facilitates immune recognition by the host. It is unclear as to which of these effects are important in antitumour activity *in vivo* (Foon, 1989; McLaughlin, 1993; Geerligs *et al.*, 1993). In animal experiments, IFN's used alone cause tumour regression with subsequent prolonged survival, but seldom result in cures. IFN's were the first human recombinant proteins used in the treatment of human cancer. α-IFN has an established role in the management of LG-NHL patients (Crowther, 1991).

IFN is known to prolong G1, G2 and S phases of the cell cycle (see Figure 1 for diagrammatic representation of the cell cycle), resulting in an overall lengthening of the cycle. It has been suggested that IFN has its effects predominantly on resting cells in the G0 phase. The antiproliferative activity of IFN is probably responsible for inhibition of lymphocyte blastogenesis (Quesada, 1983).

α-IFN is actually a family of over 30 subtypes and is produced by leukocytes (B-cells, T-cells, NK cells and macrophages) as a result of exposure to B-cell mitogens, viruses, foreign cells, or tumour cells (Foon, 1989; McLaughlin, 1993; Geerligs *et al.*, 1993). α-IFN is stable at a pH of 2 (Quesada, 1983).

α-IFN is the only IFN that has been widely used in the treatment of cancer patients, however it remains unclear whether patient responses are due to α-IFN's antiproliferative or immunomodulatory effect. It seems likely that a combination of mechanisms are involved in these anti-cancer effects that have been demonstrated. The immunomodulatory effects of α-IFN could possibly stimulate the rejection of tumour cells by the host. The stimulation of NK activity by α-IFN may also play an important role. The results could also be mediated by the induction of a cascade of cytokines or growth factors (Foon, 1989; Crowther, 1991).

Early trials were conducted using non-recombinant α-IFN, obtained from virus stimulated buffy coat leukocytes. The purity of these preparations was about 1% (10^6 U/mg protein) (Foon, 1989). See Table 8-A for a summary of clinical trials using non-recombinant α-IFN therapeutically. IFN is now produced by recombinant DNA technology and is available for therapy. This has the obvious advantage of purity and consistency in IFN activity (Parkinson *et al.*, 1993; Gaynor and Fisher, 1991). In early clinical trials using crude natural and recombinant forms of α-IFN a response rate of 33-50% could be demonstrated in LG-NHL patients (Smalley *et al.*, 1992). Using recombinant α-IFN, a response of approximately 50% in LG-NHL patients was achieved, of which most was only partial, however 5-10% did achieve complete response (Foon, 1989). See Table 8-B for a summary of clinical trials using recombinant α-IFN therapeutically.

It is a well established fact that α-IFN enhances the cytolytic capacity of NK cells *in vitro* (Kedar and Klein, 1992). Due to this fact, it was envisaged that the *in vivo* treatment of patients with α-IFN would augment NK mediated cytotoxicity *in vivo* in cancer patients against tumour cells.

α-IFN appeared to be a very promising form of treatment for use in tumours with low and potentially easily controlled proliferative rates, e.g. LG-NHL. Clemens stated in 1988, that α-IFN had definite therapeutic activity in LG-NHL patients. α-IFN is a much less impressive agent for patients with either intermediate or high-grade lymphomas. Resistance to chemotherapy does not mean that tumours are resistant to α-IFN. In fact patients who have relapsed while undergoing chemotherapy have responded to treatment with α-IFN. α-IFN has been used, either alone, or in combination with chemotherapy. An additive or synergistic effect has been observed between α-IFN's and various cytotoxic drugs in both *in vitro* and *in*

vivo studies. (Gaynor and Fisher, 1991; Foon, 1989; McLaughlin 1993; Gilewski and Richards, 1990; Cavalli 1988; Ozer *et al.*, 1983; Foon *et al.*, 1984). Two different types of recombinant α-IFN are currently in use: $alpha_{2a}$ (Roferon ®) and $alpha_{2b}$ (Intron A ®), differing by an amino acid substitution at position 23 (Gilewski and Richards, 1990; Coiffier, 1993). The combination of both chemotherapy and α-IFN may lead to more complete and durable clinical responses since α-IFN alone does not cure. Trials have therefore focused on combining therapies without increasing toxicity (Coiffier, 1993; Oken, 1992). This was confirmed with animal model experiments in which implementation of IFN treatment following chemotherapy induced both remission and prolonged survival. See Table 9 for a summary of clinical trials using α-IFN therapeutically in combination with chemotherapy. It seems appropriate to treat only those patients who have achieved a complete response after chemotherapy with α-IFN. This regimen is in accordance with that of Kedar and Klein (1992), who suggested a reduction in the tumour load by surgery followed by chemotherapy or radiation prior to the commencement of a BRM. Accumulating evidence seems to indicate that α-IFN may prolong the disease-free interval and possibly the overall survival when administered after initial cytoreductive treatment (reduction in tumour by the use of cytotoxic drugs) (Hiddemann *et al.*, 1994). The continued use of α-IFN as maintenance treatment after patient response may improve remission duration and hence possibly survival, but it is unlikely to cure patients (Rohatiner, 1991). This strategy of treating the patient with chemotherapy, followed by maintenance α-IFN maximises the effects of α-IFN at a time when the patient has minimal residual disease and biological agents are expected to be most effective. It is also at the stage following immunosuppressive therapy when the immunrestorative effects of α-IFN would be most beneficial. Unfortunately the use of α-IFN as maintenance treatment requires extended periods of treatment (McLaughlin, 1993). Kwak and co-authors (1994) comment that the α-IFN treatment appears to be simply keeping the residual disease at a subclinical level, because nearly all α-IFN treated patients relapse within a year of discontinuation of treatment.

Data from Italy, Britain and the European Organization for the Research and Treatment of Cancer group, indicate that α-IFN treatment served to prolong the period of disease free survival, after either cytoreductive chemotherapy or combination chemotherapy. The combination chemotherapy consisted of cyclophosphamide, oncovin (vincristine) and prednisone (COP). The duration of this beneficial effect was limited to the period of α-IFN treatment, usually 12 months (Hiddemann and Unterhalt, 1994). Contrary to this, however, Schuler *et al.*, 1995, state that no additional benefit is gained by combining α-IFN with chemotherapy when compared to chemotherapy alone (Horning, 1994-b).

Kwak and Longo (1996), feel that due to the long natural history of LG-NHL, a longer follow-up period is necessary, to ascertain whether the survival advantage for α-IFN treated

patients is maintained or lost, when compared to patients treated with chemotherapy alone. α-IFN treatment appears to keep subclinical levels of the disease under control, but patients experience chronic α-IFN toxicity especially fatigue and depression, without any improvement in survival (Kwak and Longo, 1996).

Absorption and clearance of α-IFN is fairly rapid following subcutaneous injection. Peak serum concentrations are found after 6-8 hours and clearance usually takes approximately 24 hours (Quesada, 1983).

TABLE 8-A. SUMMARY OF SINGLE AGENT NON-RECOMBINANT α-IFN CLINICAL TRIALS

Type of NHL	Type of α-IFN	Dose and site	Response (%)	No of patients	Reference
Non-recombinant α-IFN's					
LG-NHL	human leukocyte IFN 10^6 reference units/mg protein	5 X 10^6 U i.m. 2 X daily	PR - 3 (100)	3	Merigen *et al.*, 1978
LG-NHL	human leukocyte IFN 10^6 U/mg protein	5 X 10^6 U i.m. 2 X daily	CR - 1 (13) PR - 3 (38) MR - 3 (38) NE - 1 (13)	8	Louie *et al.*, 1981
LG-NHL	human leukocyte IFN 10^6 U/mg protein	1 - 9 X 10^6 U i.m. 1 X daily	CR - 0 PR - 3 (11) MR - 3 (11)	28	Horning *et al.*, 1985.

Results have only been shown on trials involving LG-NHL patients. Where a combination of different categories of patients were treated, results have been indicated in LG-NHL patients only.

See section 1.1.5.1. for a detailed description of response criteria.

CR = complete response
PR = partial response
MR = minimal response
NE = non-evaluable
i.m. = intra-muscular

TABLE 8-B. SUMMARY OF SINGLE AGENT RECOMBINANT α-IFN CLINICAL TRIALS

Type of NHL	Type of α-IFN	Dose and site	Response (%)	No of patients	Reference
Recombinant α-IFN's					
LG-NHL (refractory to standard treatment)	IFN-α_2 $\geq 10^8$ U/mg protein	1 - 100 X 10^6U i.m. 1 X weekly	CR - 0 PR - 1 (33) MR - 1 (33) SD - 1 (33)	3	Ozer *et al.*, 1983
LG-NHL Previously treated - now unresponsive	α-IFN	50 X 10^6 U i.m. 3 X weekly	CR - 4 (17) PR - 9 (38)	24	Foon *et al.*, 1984
LG-NHL Previously treated - now unresponsive	IFN alfa$_{2a}$ (Roferon)	Low dose 3 X10^6 U s.c. daily High dose 50 X 10^6 U i.m. 2 X weekly	Low dose CR - 0/19 PR - 5/19 (26) SD - 3/19 16) PD - 10/19 (53) High dose CR - 2/20 (10) PR - 5/20 (25) SD - 6/20 (30) PD - 7/20 (35)	39	Van der Molen *et al.*, 1990

Results have only been shown on trials involving LG-NHL patients. Where a combination of different categories of patients were treated, results have been indicated in LG-NHL patients only.

See section 1.1.5.1. for a detailed description of response criteria.

CR = complete response
PR = partial response
MR = minimal response
SD = stable disease
PD = progressive disease
i.m. = intra-muscular
s.c. = sub-cutaneous

TABLE 9. **SUMMARY OF COMBINATION α-IFN CLINICAL TRIALS**

Type of NHL	Type of I-IFN	Dose and site	Response (%)	No of patients	Reference
Combination trials - non-recombinant I-IFN					
LG-NHL (advanced) Heavily pre-treated	Human leukocyte 1-2 X 10^6 U/mg protein	6 X 10^6 U i.m. + CLB	CR - 3 (43) PR - 1 (14) NR - 2 (29)	7	Clark *et al.*, 1989
Combination trials - recombinant α-IFN's					
LG-NHL	IFN $alfa_{2b}$ (Intron A)	2 X 10^6 U s.c. alternate days + cyclo	CR - 8 (19) PR - 23 (53) MR - 8 (19) PD - 4 (9)	43	Ozer *et al.*, 1987
LG-NHL (advanced)	IFN $alfa_{2b}$ (Intron A)	3 X 10^6 U s.c. 3 X weekly + CLB	CR - 2 (20) PR - 6 (60) NR - 1 (10) SD - 1 (10)	10	Chisesi *et al.*, 1987
LG-NHL and IG-NHL	IFN $alfa_{2a}$ (Roferon A)	COPA or IFN+COPA 6 X 10^6 U i.m. 5 X days/month	COPA CR - 29% PR - 57% IFN+COPA CR - 32% PR - 54%	COPA - 127 IFN+COPA - 122	Smalley *et al.*, 1992

Results have only been shown on trials involving LG-NHL patients. Where a combination of different categories of patients were treated, results have been indicated in LG-NHL patients only. The trial conducted by Smalley *et al*, in 1992, was conducted on both low and intermediate grade patients. No distinction was made between the different grades when results were discussed, therefore these results cannot be separated into low and intermediate grade patients.

See section 1.1.5.1. for a detailed description of response criteria.

CR	=	complete response	PR	=	partial response
MR	=	minimal response	SD	=	stable disease
NR	=	no response	PD	=	progressive disease
i.m.	=	intra-muscular	s.c.	=	sub-cutaneous
CLB	=	chlorambucil	cyclo	=	cyclophosphamide
COPA	=	cyclo, vincristine, prednisone and doxorubicin			

1.1.6.1. α-IFN TOXICITY

α-IFN toxicities are directly dose-related and consist of flu-like symptoms such as fever, chills, rigors, headache, malaise, nasal congestion, myalgias, anorexia and fatigue and psychiatric disorders such as confusion/involution, depression, and also impotence, nausea, vomiting, xerostomia, mild alopecia, nail changes, development of a metallic taste sensation, paraesthesias, rash, liver injury, pulmonary embolism, pulmonary oedema, sarcoidosis, modest myelosuppression, hypotension and tachycardia. The most commonly described side-effects are flu-like in nature. At high doses, the interferons are neurotoxic and demonstrate a variety of side-effects, including behavioural changes. Various ocular side-effects have also been documented. Bone marrow depression causing neutropenia and thrombocytopenia is a serious form of toxicity. This must be borne in mind when α-IFN is

used in association with cytotoxic chemotherapy. It is imperative that patients being treated with α-IFN are warned and monitored for side-effects that may or may not be reversible (Parkinson *et al.*, 1993; Tang, 1995; McLaughlin *et al.*, 1993; Horning *et al.*, 1985; Ozer *et al.*, 1983; Kuzel, *et al.*, 1990; Crowther, 1991). In one trial, a patient with a history of coronary-artery disease had a massive myocardial infarction and died after the first dose of α-IFN. It is recommended therefore that patients with clinically important cardiac disease not receive α-IFN treatment (Foon *et al.*, 1984).

With long term treatment of α-IFN, which is recommended by most authors, the minor side effects gain importance, because phenomena such as chronic fatigue may substantially impair the quality of life. The majority of patients treated with α-IFN are relieved to have even completed one year's maintenance therapy (Ziegler-Heitbrock and Thiel, 1990; Crowther, 1991).

1.1.7. TREATMENT OF PATIENTS IN THIS STUDY

In an attempt to maximise the response for the patients on this trial, they were initially placed on combination chemotherapy of COP consisting of:- cyclophosphamide (intravenous) + vincristine (intravenous) + prednisone (oral) to reduce the bulk of tumour cells. This is a relatively non-toxic drug combination (Gaynor and Fisher,1991). Following this regimen, and if patients responded to this treatment, the patients were selected to receive either maintenance α-IFN or maintenance chemotherapy. Maintenance chemotherapy consisted of oral cyclophosphamide for a period of 2 years. α-IFN was given at a dose of 3×10^6 units by subcutaneous injection three times a week, as a form of maintenance therapy. A relatively low dose of α-IFN was selected to avoid intolerable toxicity and to obtain an acceptable rate of compliance. Synonymous with results in myeloma, hairy-cell leukaemia and chronic myeloid leukaemia, it appears that a prolonged exposure to α-IFN is most beneficial. Patients in this study group were therefore treated with maintenance α-IFN for a period of two years (Rohatiner, 1991). The α-IFN was given after the chemotherapy in an attempt to eliminate any remaining tumour cells. Maintenance immunotherapy is appealing due to the fact that lymphoma patients often have underlying immune dysfunction (McLaughlin *et al.*, 1993).

The rationale for using α-IFN as maintenance treatment was that BRM's are felt to be most effective in the elimination of minimal residual disease. Long term treatment with an α-IFN was assumed to be less likely to be associated with chronic marrow toxicity such as myelodysplasia. It was anticipated that there would be enhancement of the immune system and that some patients would remain in prolonged complete response (McLaughlin *et al.*, 1993; Crowther, 1991). Recent results published by the European Organization for the

Research and Treatment of Cancer lymphoma study group report a longer period of disease-free survival in patients treated with α-IFN as maintenance therapy after initial treatment consisting of COP. These results appear to be confirmed by an Italian and British study (Hiddemann and Unterhalt, 1994).

1.1.8. ADVERSE HAEMATOLOGICAL COMPLICATIONS OF CHEMOTHERAPY

In patients treated with chemotherapy, no direct effect is observed on mature cells in either the blood or bone marrow, with the exception of the lymphocyte. As a result of this, the life span of peripheral blood cells determine when a particular cytopenia will develop. Neutrophils survive for about 10 hours, platelets for about 10 days and red blood cells for about 120 days. Cytotoxic agents can be categorised as:- cell cycle phase specific, when cells in a particular phase of the cell cycle are targetted, or:- phase non-specific, when no particular phase of the cell cycle are targetted (see Figure 1 for a diagrammatic representation of the cell cycle). Cycle-nonspecific drugs may kill cells that are not actively dividing and are effective in tumours in which the growth fraction is low. Phase-specific agents exert their anti-neoplastic activity in a particular phase of the cycle, and such drugs are more effective among tissues that exhibit a high growth fraction. The COP regimen used for treating NHL patients on this study is considered a relatively non-aggressive form of treatment (Bodensteiner and Doolittle, 1993; Dorr and Fritz, 1982). A combination of drugs, usually with different killing mechanisms are selected. This treatment protocol maximises the killing of tumour stem cells and prevents tumour resistance to a single form of therapy. Cyclophosphamide is not in itself an alkylating agent, but is converted by the liver into an alkylating compound which is phase non-specific (Bodensteiner and Doolittle, 1993; Dorr and Fritz, 1982; Babior and Stossel, 1994). Cell cycle-nonspecific drugs such as cyclophosphamide will destroy all haemopoietic cell production. Only the pool cells and post-mitotic maturing cells in the bone marrow will be spared. Cyclophosphamide treatment is usually well tolerated. Vincristine is one of the active principles of the vinca alkaloids and is a M-phase-specific drug. The degree and duration of bone marrow suppression depends on the type of agent used. Cell cycle-specific agents tend to cause early myelosuppression with rapid marrow recovery compared with non-phase-specific agents. The mode of action and toxicity of prednisone appears similar to that of other alkylating agents. The prednisone regimen for NHL generally produces less severe haematological toxicity than for other neoplasms. Toxicity includes myelosuppression which is characterised by dose-dependent leukopenia and thrombocytopenia. There also appears to be no cross-resistance between prednisone and the other alkylating agents such as cyclophosphamide (Bodensteiner and Doolittle, 1993; Dorr and Fritz, 1982; O'Reilly and Connors, 1992).

When using peripheral blood counts to monitor dosage and frequency of chemotherapy, it is essential to remember that changes in the peripheral blood cell counts lag behind changes in the bone marrow production pools. If the leukocyte count remains in the normal range for patients treated with chemotherapy, this should be increased, until treatment causes mild myelosuppression. This is to ensure that a sufficient amount of the drug is being absorbed (Bodensteiner and Doolittle, 1993; Dorr and Fritz, 1982; O'Reilly and Connors, 1992).

1.2.0. NATURAL KILLER CELLS

1.2.1. MORPHOLOGY AND COMPOSITION OF NK CELLS

Human natural killer (NK) cells are a small population of lymphoid cells of distinct lineage which differ from T and B lymphocytes in that they do not express receptors for surface immunoglobulin or T cell antigen receptors (TCR). NK cells account for approximately 10-15% of peripheral blood mononuclear cells (PBMC). At least 70% of human PBMC large granular lymphocytes (LGL) have been shown to have NK activity (Reynolds and Wiltrout, 1989). Both cell number and cytotoxicity varies among normal individuals (Gong *et al.*, 1994: Lotzová, 1991; Moretta *et al.*, 1994-a). NK cells are generally larger than resting T or B lymphocytes and display the typical LGL morphology, containing intracytoplasmic azurophilic granules and have a high cytoplasmic to nuclear ratio (Roitt *et al.*, 1987; Moretta *et al.*, 1994-a). NK cells may also be of medium size and agranular, but this may be related to the NK cell stage of differentiation (Lotzová, 1993). Granules appear to play a role in cytolytic activity, as granular content correlates with cytolysis (Shibuya *et al.*, 1993, Lebow *et al.*, 1993). Using electron microscopy, the LGL appear as medium sized lymphocytes with round or indented nuclei, condensed chromatin, prominent nucleoli and extended golgi apparatuses.

1.2.2. FUNCTION OF NK CELLS

NK cells are so named, because they have spontaneous or natural cytotoxicity against cells infected with bacteria and viruses or tumour cells, without prior *in vitro* or *in vivo* activation. These cells are therefore important in innate immunity (Caldera *et al.*, 1992; Kos and Engleman, 1996). The cytolytic function of NK cells has been described as natural killer activity (NKA) and differs from the immunologically specific lytic activity of cytotoxic T lymphocytes (CTL) in that it is not major histocompatibility complex (MHC) restricted, and does not follow immunology rules of selectivity, specificity and memory (Reynolds and Wiltrout, 1989).

Not all NK cells bind to targets and of those that do bind not all are cytotoxic (Lebow *et al.*, 1993). NK cells have been proposed as the first line of defense against tumour cells (Caldera *et al.*, 1992). These cytotoxic effects can be observed against a variety of tumour cells, both non-immunogenic as well as immunogenic (Reynolds and Wiltrout, 1989).

Often it is necessary for the target cell to be precoated with specific IgG prior to the killing of the target cell. This lytic process is known as antibody-dependent cellular cytotoxicity (ADCC) (Abbas *et al.*, 1994). NK cells, together with the humoral immune system, are responsible for the elimination of target cells via ADCC (Metes *et al.*, 1994).

NK cells are rapidly recruited to sites of inflammation by their ability to respond to chemokines and inflammatory mediators. NK cells also express many adhesion receptors which facilitate margination, extravasation and migration of these cells (Palmieri and Santoni, 1997).

NK cells may also play a large role in maintaining the homeostasis of the haemopoietic compartment. This hypothesis was confirmed by *in vitro* human studies that indicated NK cells displayed control over the colony-forming potential of both erythroid and myeloid precursors. Evidence also appears to imply that proliferation, differentiation, and functions of some members of the lymphoid compartment could be controlled by NK cells. The exact mechanisms by which NK cells regulate this activity is unclear, although some of these effects appear to be related to cytokine production (Lotzová, 1993; Perussia, 1991; Trinchieri, 1992).

1.2.3. NK CELLS AND TISSUE DISTRIBUTION

NK cells have been identified in various tissue compartments other than the peripheral blood, e.g. lungs, red pulp of the spleen, intestinal mucosa, liver and peritoneal cavity. NK cytotoxicity has also been detected in human tonsils. NKA levels are highest in peripheral blood and spleen. In some tissue, it is necessary to activate cells before cytotoxicity is detected. NK cells are present in small numbers in lymph nodes, although they do not travel through the lymphatic circulatory system. NK cells do not recirculate between blood and lymph as T cells do (Lotzová, 1991; Warren, 1996). Spontaneous NKA in lymph nodes is relatively low, but this cytolytic activity can be stimulated. Stimulation of NKA and maintenance of these levels, may contribute to the treatment of cancer patients (Reynolds and Wiltrout, 1989).

1.2.4. CONTROL OF NK FUNCTION BY NON-LYMPHOID CELLS

Since red blood cells (RBC) form the most abundant component of peripheral blood, NK cells as well as other lymphocytes are continually in contact with RBC's during circulation. RBC's have been found to enhance NK cytotoxicity *in vitro*. This enhanced cytotoxicity was observed with two target cell lines viz. the erythroleukemic cell line, K562 and the histiocytic cell line, U937. Only intact RBC's and not RBC lysates were found to enhance cytotoxic activity. Evidence suggests that a unique RBC protein may be responsible for this augmentation (Shau *et al.*, 1993).

Polymorphonuclear neutrophils account for the most abundant leukocytes in the peripheral blood. Polymorphonuclear neutrophils have been found to inhibit NK cytotoxicity *in vitro*. In the presence of TNF, which is a potent polymorphonuclear neutrophil stimulator, this suppression was found to be even greater. Cell-cell contact was necessary for this inhibition, a phenomenon that was observed for intact and viable polymorphonuclear neutrophils only (Shau *et al.*, 1993).

It seems likely that both RBC's and polymorphonuclear neutrophils play an important role in the regulation of NK function *in vivo* (Shau *et al.*, 1993).

1.2.5. NK CELLS AND SURFACE MARKER EXPRESSION

There is currently much research being undertaken into NK cells and antigen expression. Some of these NK cell receptors are essential in cytotoxicity, while others are important in adhesion, or as cytokine receptors (Yokohama, 1995).

NK cells are characterised by expression of Cluster of Differentiation antigen (CD) CD56 and CD16 (predominantly but not exclusively present in NK cells - this marker can also be found on macrophages, neutrophils and a subset of eosinophils) and lack of CD3 expression. CD3 is always expressed in association with the TCR. NK cells do not express the α/β or γ/δ genes of the TCR, however, they may express the ε- or ζ- chain of the CD3 molecule.

CD56 is a typical but not exclusive NK cell molecule. This molecule is also present on T cell subsets and neural and muscle tissues. NK cells express a low-affinity receptor for the Fc portion of IgG, called FcγRIII or CD16. NK cells are capable of binding to IgG immune complexes via CD16 molecules on their surface. This interaction activates the NK cell lytic mechanism and induces production of lymphokines by the activated cells. Functionally CD16 is important for mediation of antibody-dependent cellular cytotoxicity.

NK cells can be distinguished from T cells by their lack of expression of CD3, CD4 and CD5 cell surface molecules and of T cell receptor chains $\alpha,\beta,\gamma,\delta$. In addition NK cells display *in vitro* cytotoxic activity against NK-sensitive targets such as the erythroleukaemic human cell line, K562 (Moretta *et al.*, 1994-a; Gong *et al.*, 1994; Spits *et al.*, 1995; Trinchieri and Valiante, 1993; Lotzová, 1993; Pollack, 1993). CD2, the low-affinity rosette receptor, is also present on most mature NK cells and may also be important in target cell interaction (Lotzová, 1991; Trinchieri, 1992). NK cells may also express CD8 and CD57 (Warren, 1996). NK cell progenitors *in vitro* that lack CD56 expression also lack cytotoxic activity against NK sensitive targets. On further culture these CD56- cells gained both CD56 expression and cytotoxicity. There appears to be therefore an association between the cytotoxic activity of NK cells and the expression of CD56 (Spits *et al.*, 1995). CD56 may also be important in NK cell interaction with targets (Lotzová, 1993).

CD7, a 40 kilodalton (kDa) glycoprotein present on mature NK cells may play a role in adhesion and cell activation (Miller JS *et al.*, 1994).

Resting NK cells do not express HLA-DR antigens (class II MHC), but when activated they often express HLA-DR and transferrin receptors.

NK cells activated by IL-2 or α-IFN express CD69 activation antigens and increased expression of this marker correlates with cytolytic activity. CD69 is upregulated in binder and killer cells compared to free cells (Bonavida *et al.*, 1993; Jewett and Bonavida, 1995).

1.2.6. NK CELL DEVELOPMENT

It has been established that a functional thymus is not necessary for maturation of NK cells, however it is possible that NK differentiation could occur in the thymus (Moretta *et al.*, 1994-a; Spits *et al.*, 1995; Moretta *et al.*, 1994-b).

It is possible that the thymus is colonised by multipotential haemopoietic progenitor cells that are distinct from stem cells. These progenitor cells have the potential to develop into haemopoietic non-T cells within the thymus. Athymic mice have normal numbers of NK cells in the peripheral blood (Spits *et* al., 1995). It is likely that the bone marrow is the main site for the development and differentiation of NK cells, although both NK cell number and cytotoxicity in the bone marrow of healthy donors is low (Spits *et al.*, 1995; Lotzová 1991; Haller *et al.*, 1977). See Figure 2 for a diagrammatic representation of production and differentiation of NK cells. See Table 10 for morphological characteristics of development of the NK lineage.

A description for the development of NK cells now follows. Large proliferating progenitor NK cells in the bone marrow, give rise to less rapidly proliferating precursor NK cells. The presence of stromal cells and growth factors is essential for this to take place. Precursor NK cells exhibit some phenotypic and morphological characteristics of NK cells. These precursor cells divide and differentiate in the bone marrow into phenotypically recognisable primary NK cells. It is important to note the progressive decrease in cell size and the acquisition of typical NK cell surface antigens as differentiation proceeds. These primary NK cells are relatively small lymphocytes (about 8 μm) and are agranular as compared to mature NK cells. Primary NK cells are not in cell cycle and have low lytic activity and express the phenotype of resting NK cells. Primary NK cells are able to respond to both interferon and IL-2, but not as well as mature NK cells. These cells leave the bone marrow and undergo further maturation in peripheral sites. Peripheral NK cells are activated by agents such as interferon to undergo proliferation in response to stimuli. It should be noted that although peripheral NK cells are highly activated by interferon, less mature NK precursors seem to be refractory to interferon (Pollack, 1993).

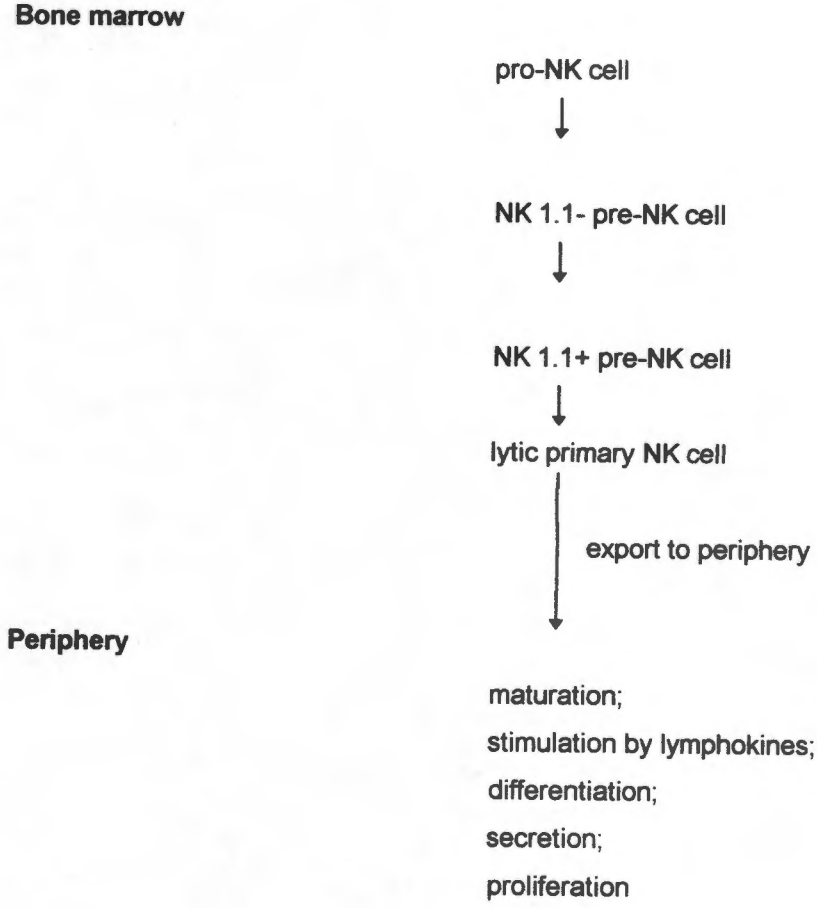


FIGURE 2. Production and differentiation of NK cells
(based on data from the mouse) (Pollack,1993).
Pre = precursor Pro = progenitor NK1.1 = NK equivalent to either CD16 or CD56 in humans

The presence of IL- 2 is required for *in vitro* or *in vivo* culture of bone marrow derived NK cells, suggesting that NK progenitors have IL-2 receptors (IL-2R) (Miller *et al.*, 1992; Lotzová *et al.*, 1993; Silva *et al.*, 1994; Lotzová *et al.*, 1991, Shibuya *et al.*, 1993).

It is possible that NK cells may develop in other organs such as spleen, liver, or blood, but as yet this issue has not been resolved (Spits *et al.*, 1995).

TABLE 10. **MORPHOLOGICAL CHARACTERISTICS OF THE DEVELOPMENT OF THE NK LINEAGE**

(based on data from the mouse) (Pollack, 1993).

	PRO-NK	PRE-NK	PRIMARY NK	PERIPHERAL NK
Size	large	large-medium	small	varies
% in cycle	75	25	0	varies
Lytic	-	-	+	++ to ++++
IL-2 responsive	-	+	+	+
IFN responsive	-	-	(+)	+

pro = progenitor pre = precursor

Both cytolytic NK cells and/or T cells have been generated from CD34+ or CD34- bone marrow cells, depending on the *in vitro* conditions (Shibuya *et al.*, 1993; J.S. Miller JS *et al.*, 1994; Miller *et al.*, 1992; Lotzová *et al.*, 1993). See Figure 3 for the development of T and NK cells. It appears therefore, that CD34+ cells are primitive progenitors for either NK cells or T cells and as these cells differentiate, they lose the CD34 expression. These NK cells then acquire expression of CD2, CD16 and CD56 antigens (which are usually found on mature NK cells) as well as gaining cytolytic activity (Spits *et al.*, 1995; Lotzová and Savary, 1993). The CD34+ population display the typical blast morphology and the mature NK cells the LGL morphology, with a progressive decrease in cell size as maturation takes place (Lotzová and Savary, 1993; Pollack, 1993). Bone marrow NK cells have a rapid turnover time (Pollack, 1993). NK cells have also been cultured from CD34+ populations depleted of the CD34+, CD33+ subset, which represents the committed myeloid progenitor, indicating that these cells are not required for NK cell generation (Lotzová and Savary, 1993).

It is possible, that in addition to IL-2, other cytokines are necessary in the development of NK cells and that as these progenitor cells differentiate, they in turn provide other essential cytokines. It has been shown that TNF-alpha enhances the IL-2 driven production of NK cells (Lotzová and Savary, 1993).

NK cells have not been found to lyse normal autologous cells which appears to indicate that an NK cell receptor can identify at least one self MHC class I allele. The molecular mechanisms of this, however, remain unknown (Spits, *et al.*, 1995).

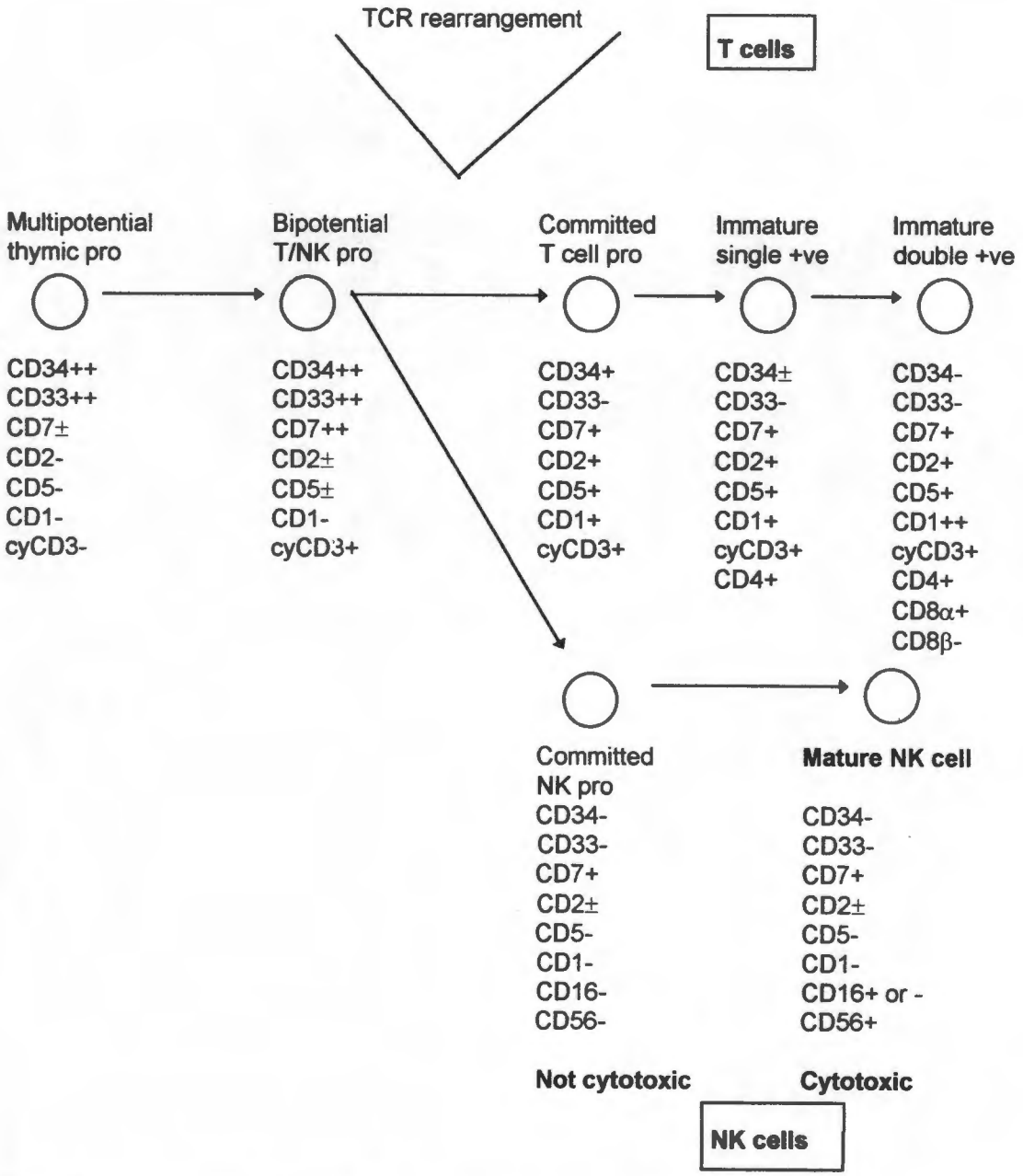


FIGURE 3. Model of T and NK cell development in the thymus (Spits *et al.*, 1995).

Pro = progenitor cyCD3 = cytoplasmic CD3

1.2.7. CYTOKINES AND NK CELL DEVELOPMENT

Table 11 summarises the effects of cytokines and growth factors on production of NK cells from bone marrow. IL-2 (produced exclusively by activated T-cells), is a potent stimulator of NK cells, causing not only proliferation, but cytokine production and increased cytotoxicity (Naume and Espevik, 1994; Warren, 1996). Various cytokines appear to play either positive or negative regulatory roles during the production of NK cells (Pollack,1993). IL-3 appears to

inhibit the IL-2 driven generation of NK cells from bone marrow *in vitro* (Pollack,1993). IL-4 either inhibits or stimulates the production of NK cells, depending on the co-stimulus used and the activation state of the NK cells (Pollack, 1993; Naume and Espevic, 1994). The reported effect of IL-6 on NK cells has been contradictory, but this cytokine appears to have stimulatory effects on NK cells. IL-6 also induces adhesion molecules, TNF-alpha and TNF-beta cytokine production, and moderate proliferation in NK purified cells (Naume and Espevik, 1994; Ortaldo *et al.,* 1995). Controversy exists as to the role of IL-7 in the development of NK cells. Pollack (1993) states that IL-7 does not appear to play a crucial role in the development of NK cells, whereas Spits and co-workers feel that evidence points to IL-7 as being involved in the development of NK cells (Spits *et al.*, 1995). Ortaldo and co-authors (1995), report that IL-7 activates NK cytotoxicity. IL-12, also known as NK cell stimulatory factor or cytotoxic lymphocyte maturation factor, which is produced by monocytes and B cells, has several effects on NK cells including:- stimulation of proliferation, increase in IFN-γ production, enhancement of adhesion molecule expression, increase in granule content and augmentation of cytotoxicity (Klein and Mantovani, 1993; DeBlaker-Hohe *et al.*, 1995; Naume and Espevik, 1994). α-IFN and γ-IFN has been found to activate NK cytotoxicity (Ortaldo *et al.,* 1995). IL-15 can support human NK survival *in vitro* in the absence of serum or any other growth factors, possibly by the prevention of early apoptosis. Il-15 appears, at least in part, to be able to mimic the bone marrow environment in NK cell differentiation. IL-15 is also able to activate cytotoxic activity and cytokine production by human NK cells, as well as inducing proliferation of NK cells. Furthermore, as IL-15 appears to be constitutively expressed in various cells, including monocytes/macrophages, it may play an important role in the regulation of NK cell function. The 3-D structure of IL-15 is similar to that of IL-2 and both of these cytokines display similar functions in their ability to activate both T and NK cells (Carson *et al.,* 1997; Leclercq *et al.,* 1996; Salvucci *et al.,* 1996; Puzanov *et al.,* 1997). Murine IL-15 caused the maturation and differentiation of nonlytic murine NK cells *in vitro* (Puzanov *et al.,* 1996).

NK precursors appear refractory to the stimulatory effects of α-IFN or β-IFN *in vitro*, whereas mature PBMC derived NK cells can be highly activated by IFN. Both γ-IFN and TNF-α appear to play an important role in the primary production of NK cells (Pollack, 1993).

TABLE 11. **EFFECTS OF CYTOKINES AND GROWTH FACTORS ON PRODUCTION OF NK CELLS FROM BONE MARROW**

(based on data from the mouse) (Pollack, 1993).

CYTOKINE	*IN VIVO*	*IN VITRO*	EFFECTS ON NK CELL PRODUCTION
IL-2	+		increases number of $ASGM_{1+}$ cells (NK cells) and NKA in bone marrow
		+	induces generation of NK cells from BMC
IL-1	+		augments IL-2 induced production in combination with TNF
		+	no effect alone; augments IL-2 dependent generation of NK
IL-3	+		decreases frequency of IL-2 responsive precursors
		+	decreases IL-2 dependent generation of NK cells when added early
			enhances IL-2 dependent generation of NK cells at low doses of IL-2
IL-4		+	decreases generation of NK cells at high doses of IL-2
			increases generation of NK cells at low doses of IL-2
IL-5		+	no effect
IL-6		+	no effect
IL-7		+	no effect
IFN ★		+	IFN-α/β has no effect on precursors; little effect on primary NK cells
			IFN-γ augments response to IL-2
			anti-IFN blocks IL-2 induced differentiation of BMC
CSF-1		+	augments IL-2 dependent generation of NKA
GM-CSF		+	inhibits IL-2 dependent generation of NK cells
TNFα		+	preculture in TNF augments response to IL-2; anti-TNF blocks

NKA = NK activity BMC = bone marrow cell
CSF = colony-stimulating factor
GM-CSF = granulocyte/macrophage colony-stimulating factor
★ Although NK cells in the peripheral blood can be highly activated by IFN, less mature NK cells appear refractory to IFN.

1.2.8. THE COMPOSITION OF NK CELL GRANULES

Granules isolated from cytotoxic lymphocytes have been found to be cytolytic towards some nucleated cells, as well as to RBC's. It has been hypothesised that these granules are involved in NK cell-mediated killing (Lowin *et al.*, 1994).

All activated CTL's and cytolytic NK cells contain approximately 30-50 lytic granules. Based on electron microscopic studies there appears to be three types of granules which have been designated type I, type II and intermediate. Proteins present in killer cells can be divided into three groups, perforin, granzymes and T1-A (Griffiths and Argon, 1995). Perforin (cytolysin or pore-forming protein) is a 70 kDa membraneolytic protein and the granzymes (granule enzymes) are serine esterases. The third group of protein, a poly(A)-binding protein, is TIA-1 and its relative TIAR which are RNA binding proteins, and could be a natural target of granzymes (Radoševic *et al.*, 1995-a; DeBlaker-Hohe *et al.*, 1995; Yagita *et al.*, 1992; Berke, 1994; Griffiths and Argon, 1995; Greenberg and Litchfield, 1995; Lowin *et al.*, 1994; Liu *et al.*, 1995).

Four human granzymes have been identified (granzyme A, B, H and possibly 3) (Lowin *et al.*, 1994). Granzymes A and B have been described and cloned. Granzyme A possesses trypsin-like specificity. Granzyme B is the most abundant granzyme present in cytoplasmic granules and binds proteins (Smyth and Trapani, 1995). Granzyme B is most likely involved in a proteolytic cascade leading to apoptosis (Lowin *et al.*, 1994). Another function of granzymes could be cytostasis:- the prevention of cellular proliferation without death (Lowin *et al.*, 1994).

Another component of the granules are proteoglycans which are not restricted to killer lymphocytes, and may serve a role in the compression of proteins inside the granules to form a higher density. Proteoglycans may also prevent autolysis, by enveloping granular proteases. Chondroitin sulfate is the predominant proteoglycan in these lytic granules and appears to play important structural roles and also maintain the proteins in an inactive state until a pH change occurs as a result of granule exocytosis (Griffiths and Argon, 1995; Lowin *et al.*, 1994; Berke, 1994; Trapani and Smyth, 1993).

Lysosomal/endosomal enzymes, receptors and membrane proteins are also present in lytic granules. Soluble lysosomal hydrolases and lysosomal membrane proteins reside in the granules (Griffiths and Argon, 1995; Lowin *et al.*, 1994; Berke, 1994; Trapani and Smyth, 1993).

Calreticulin, a calcium-binding 46 kDA protein, is also present and is possibly involved in the binding of calcium. Calreticulin may also play an important role as a Ca^{2+} buffer and in the protection of perforin, which is inactivated by low concentrations of calcium. It has been proposed by Bleackley and co-workers (1995), that calreticulin may be important in accompanying perforin to the granules in two ways; as a chaperone ensuring that perforin remains inactive; in protecting perforin from coming in contact with free Ca^{2+}. These authors also propose that calreticulin may be important in ensuring that sufficient amounts of Ca^{2+} are available on granule exocytosis (Griffiths and Argon, 1995; Lowin *et al.*, 1994; Berke, 1994; Trapani and Smyth, 1993; Bleackley *et al.* 1995).

Lytic granules clearly function as secretory organelles both storing and secreting the lytic proteins in a highly specific manner (Griffiths and Argon, 1995; Lowin *et al.*, 1994; Berke, 1994; Trapani and Smyth, 1993).

All NK cells are positive for perforin (97-99%) when staining with an anti human perforin monoclonal antibody (Konjevic *et al.*, 1995). Perforin expression has been found to correlate with cytotoxicity. In the circulating nonactivated lymphocyte population, perforin and granzymes are only expressed in NK cells and gamma/delta T cells. Reduced cytolysis is the result of treatment with granzyme inhibitors. DNA breakdown is also inhibited by the use of serine esterase inhibitors. Granzymes appear to act within the target cell as either activators of cellular degradation, or directly in DNA fragmentation. Granzyme A binds to nuclear proteins, particularly nucleolin (Lowin *et al.*, 1994).

Not all molecules present in these granules are involved directly in cytolysis, but could serve a role in maintaining the granule structure, as well as control in activation of lytic molecules to prevent autolysis (Lowin *et al.*, 1994).

1.2.9. NK CELLS AND THE MECHANISMS OF CYTOTOXICITY

The exact mechanisms by which NK cells recognise and kill their targets has still not been totally clarified and remains the subject of intense research and debate (Berke, 1995). In principle the cytolytic function consists of three distinct steps:- Recognition and binding of tumour cells, resulting in stable lymphocyte-target conjugates; Subsequent delivery of lethal hit and dissociation and; Recycling of the lymphocyte while the target undergoes lymphocyte-independent lysis (Lotzová, 1993; Bonavida, *et al.*, 1993). Binder and killer cells appear to be poor recyclers (Bonavida, *et al.*, 1993). Berke (1994) states that NK cells are capable of recycling, but, unlike most CTL, which are able to lyse targets for at least two or more cycles without necessitating reactivation, NK cells require reactivation with lymphokines. One of the most important steps during cytotoxicity is the conjugate formation between target and NK

cell. This is mediated by receptor-ligand interactions between lymphocyte adhesion molecules and target cell ligands and allows the NK cell to recognise whether or not the target is to be killed. Secretion of granules into the limited area of cell contact ensures a high degree of target specificity during the killing process (Radoševic *et al.*, 1995-a; Radoševic *et al.*, 1995-b).

There appear to be two mechanisms of cytotoxicity:- The perforin-dependent pathway (Figure 4) mediated by the secretion of perforin (calcium ions are required for both the release of perforin from killer lymphocytes and for its pore-forming activity); The non-secretory pathway which is Fas-dependent, resulting in apoptosis (Kägi *et al.*, 1995; Liu *et al.*, 1995). The literature reports researchers with diametrically opposed views on whether or not NK cells kill by osmotic lysis (secretory) and/or apoptosis (non-secretory). Greenberg and Litchfield (1995), state that it is possible in NK mediated cytolysis that target cells demonstrate features consistent with both apoptosis and necrosis. Palmieri and Santoni (1997) agree with these previous authors, that in response to target recognition, NK cells display both secretory and non-secretory lytic methods. Lowin and co-workers (1994), state that NK cells kill target cells, not as a result of osmotic lysis, but rather as a result of apoptosis, but these authors also quote other researchers who have demonstrated cytotoxicity by osmotic lysis. Other authors feel that it is possible that in NK mediated cytotoxicity, Fas-based killing plays a role as well as the perforin/granule exocytosis model, because in experiments using perforin-free NK cells, target cells were lysed. These authors also feel that due to the heterogeneity of NK cells, it is possible that different lytic mechanisms play a role in cytotoxicity (Berke, 1994; Lowin *et al.*, 1994). Radoševic and co-authors state that cytolysis occurs in the absence of calcium, which is necessary for perforin activity and that cytolysis can also occur without visible degranulation (Radoševic *et al.*, 1995-b). Other authors estimate that Fas-based killing accounts for approximately 25% of the lysis in short-term assays (Lowin *et al.*, 1994). Perforin has also not been detected in the medium following cytotoxic assays (Berke, 1994). Perforin has been proposed as playing a central role in cytotoxicity due to circumstantial evidence. Ring-like lesions have been found in the lipid bilayer on target cells after NK cytotoxicity. Cytoplasmic granules, isolated from NK cell lines, were found to be cytotoxic to a number of target cells by producing pores on the target cell membranes in precisely the same manner as intact NK cells. Perforin has been isolated from cytoplasmic granules both *in vitro* and *in vivo* (Yagita *et al.*, 1992; Smyth *et al.*, 1994). In experiments using perforin-knockout mice, it has been found that perforin is necessary for the primary lytic mechanism, but that perforin alone is incapable of causing DNA fragmentation. Granzyme B has been shown to be essential in inducing DNA degradation in target cells (Greenberg and Litchfield, 1995; Smyth and Trapani, 1995; Heusel *et al.*, 1994; Shresta *et al.*, 1995). There have been conflicting reports on the entry of granzyme B into the target cell. Shi and co-workers (1997), report that granzyme B is able to enter the cell

cytoplasm in the absence of perforin. Froelich *et al.* (1996), however, state that in the absence of perforin, granzyme B is unable to enter the cytoplasm. Shi *et al.*, (1997) report that granzyme B remains inactive unless perforin is present, as perforin is necessary for apoptosis and translocation of the granzyme B to the nucleus.

Cytoplasmic granules in NK cells were seen to be positioned near susceptible target cells after conjugation. The exocytosis of granule contents, including perforin, has been found in the space between the killer and target cell membrane. Release of granule contents, such as serine esterases and proteoglycans was detected on conjugation (Yagita *et al.*, 1992). By the use of protease inhibitors, cytotoxicity can be completely abrogated, thus postulating a role for granzymes in cytotoxicity, although granzymes on their own are unable to cause cytolysis. If target cells were treated with a sub-lytic concentration of perforin, together with granzyme B, it was found that granzyme B had DNA-fragmenting activity. If granzyme B acts on a cytoplasmic substrate, it could possibly release a DNase from actin, thus allowing the DNase to enter the nucleus and cause DNA degradation. Thus, although DNA fragmentation is not necessarily required for cell death, it occurs rapidly during perforin and granzyme mediated cell death. It has also been shown that if perforin and granzyme A are both added to target cells, DNA fragmentation can be induced. Perforin thus provides access for granzymes into target cells, but it appears that perforin also contributes in some way to apoptosis (Griffiths and Argon, 1995; Smyth and Trapani, 1995; Greenberg and Litchfield, 1995). Nucleolin has been identified as a possible intracellular target for granzyme A, as it binds the protease and it could serve as a shuttle to transport this granzyme to the nucleus of the target cell. Granzymes could possibly affect the cytoskeleton of the target cell, or could increase the damage caused by perforin without itself causing damage to the membrane, by increasing Ca^{2+} levels within the cell, or by preventing repair mechanisms. Granzymes could, on the other hand, have no affect on the membrane at all, but merely serve as a necessary component for perforin processing (Greenberg and Litchfield, 1995). Ebnet and co-workers (1995), showed, using granzyme A-deficient mice, that granzyme A does not play a primary role in cell-mediated cytotoxicity by NK cells, as was previously thought.

Montel and co-workers (1995-a), have reported that a human NK-like cell line (YT-INDY), as well as purified fresh human NK cells utilised the Fas lytic pathway and the granule-mediated pathway. Fas-mediated cytotoxicity is Ca^{2+} independent, unlike the perforin/granule model and utilises the interaction of Fas antigen (APO-1 or CD95) on the target cell with Fas ligand on the cytotoxic cell, triggering apoptosis. This Fas-mediated cytotoxicity could be partially inhibited by blocking with anti-Fas monoclonal antibody, whereas inhibition of granzyme B or perforin had no effect on this cytolytic pathway (Montel *et al.*, 1995-a; Montel *et al.*, 1995-b). Although Oshimi and co-workers (1996), found Fas ligand expression on freshly isolated human NK cells, Eischen and Leibson (1997) feel that this finding may be incorrect, due to

technical methods employed. Eischen and Leibson (1997) report that human NK cells require activation for FasL expression. Oshimi and co-workers (1996) further report that in the presence of Ca^{2+}, the percentage of apoptotic cells is profoundly reduced by anti-Fas mAb. This would suggest that the Fas-mediated pathway as well as the perforin/granzyme pathway is significant in NK mediated cytotoxicity. Vujanovic and co-authors (1996), also report that nonactivated NK cells are capable of killing via necrosis (secretory) and apoptosis (non-secretory). These authors state that the two methods of cytotoxicity are mutually exclusive and complementary. Smyth and co-authors feel that perforin-mediated cytotoxicity appears to be the primary method of target cell lysis by NK cells (Smyth *et al.*, 1994).

Lymphokine activated killer (LAK) cells (NK cells cultured with IL-2 *in vitro* are called LAK cells), apparently lyse target cells without granule exocytosis, resulting in controversy as to the key role of perforin in cytotoxicity, although it must be borne in mind that this is an *in vitro* observation only (Yagita *et al.*, 1992). LAK cells have been shown to express low level of FasL (Eischen and Leibson, 1997). LAK cells generated from perforin and Fas ligand deficient mice indicated that these were the only molecules important in short term cytotoxicity assays. In long term cytotoxicity assays (16-24 hours), these LAK cells generated from knockout mice had significant cytotoxicity which could be attributed to TNF. TNF is both membrane bound and soluble and constitutively expressed by LAK cells. With the addition of anti-TNF antibodies this LAK mediated cytotoxicity is completely inhibited. LAK cells are armed with at least three cytotoxic molecules:- perforin, Fas ligand and TNF (Lee *et al.*, 1996).

Peripheral blood NK cells expressed perforin mRNA in both stimulated and unstimulated cells (Yagita *et al.*, 1992). Active granzyme B and granzyme A are present in the nuclei of LAK cells, but perforin on the other hand, was not detected (Smyth *et al.*, 1994).

Radoševic and co-workers, (1995-b) found that the intracellular concentration of calcium increases in target cells that are attacked by cytotoxic cells and was sometimes found to be essential for effective cytolysis. Calcium, at a high enough concentration, may trigger a destructive pathway in the target cell. Changes in membrane potential of target cells have previously been found to be associated with the killing mechanism. These authors found that target cells under attack by cytotoxic cells responded in one of two ways. Target cells either die immediately (on average within 3 minutes), as a result of the membrane impermeability breaking down, or target cells appear to repair the initial membrane damage and survive. These two reactions may correlate with two separate methods of cytolysis utilised by NK cells. It is possible that these two different responses correspond to a greater susceptibility of target cells, or differing killing capacity of NK cells. The action of perforin probably

accounts for target cells that die immediately, resembling the colloid osmosis theory (Radoševic *et al.*, 1995-b). Clearly there is no consensus of opinion.

In a recent review article, by Kägi and co-workers (1996), it was stated that NK cells utilise the perforin-mediated cytotoxicity pathway exclusively. NK cells form conjugates with target cells and cytolytic granules are excreted during this interaction, inducing the formation of lytic pores in the target cell membrane. This has led to the formation of the granule exocytosis model for NK cytotoxicity, which is further confirmed by the isolation and cloning of perforin from cytolytic lymphocytes. Perforin is incapable of causing DNA fragmentation, but granzymes entering target cells through pores caused by perforin, trigger an internal disintegration pathway resulting in DNA fragmentation. Kägi and colleagues (1996), state that the mechanisms of lymphocyte-mediated cytotoxicity has been elucidated by the use of perforin-deficient mice and state that NK cells utilise the perforin-mediated pathway of cytotoxicity exclusively.

There now follows a model of NK cytotoxicity which incorporates evidence from several authors. When an NK cell recognises a target cell, such as a tumour cell, and binds the cell, this stimulates reorganisation of the NK cell cytoskeleton and secretory apparatus. Within approximately five minutes, the microtubule organising centre and the golgi complex rearrange such that the lytic granules concentrate at the site near the bound target cell. The granules move along microtubules using a kinesin-like motor, thereby concentrating at the area of conjugation (Griffiths and Argon, 1995; Berke, 1994). Thereafter, in the presence of Ca^{2+}, granules fuse with the plasma membrane, releasing the granule contents into the space between target and NK cell, thereby delivering the "lethal hit". It has been suggested that perforin polymerises in the presence of calcium to form transmembrane pores, which appear as ring-like tubular lesions, with an inner diameter of approximately 16 nm (ranges 5-20 nm) in the target cell membrane. It has been suggested however, that the functional diameter is approximately 50 ångström units. It is unclear whether perforin plays a direct lytic role in cytolysis, or whether it functions in a more indirect manner by serving as a conduit for other granule mediators. It appears that perforin is involved in the indirect manner, but these methods are not necessarily mutually exclusive. The pores allow the release of cytoplasmic molecules from the target cell and the influx of ions, water and cytoplasmic granule contents such as serine esterases from the NK cell, causing membrane blebbing, DNA fragmentation and apoptotic nuclear morphology within minutes (Radoševic *et al.*, 1995-a; DeBlaker-Hohe *et al.*, 1995; Yagita *et al.*, 1992; Berke G, 1994; Konjevic *et al.*, 1995; Smyth and Trapani, 1995; Greenberg and Litchfield, 1995; Smyth *et al.*, 1994; Liu *et al.*, 1995). The pores are of sufficient size to allow the passage of dyes and even large proteins into the target cell (Lowin *et al.*, 1994). Apoptotic bodies are rapidly phagocytosed by adjacent cells. DNA fragmentation is visible on agarose gels as the so-called DNA ladder.

The target cell undergoes osmotic changes as a result of membrane damage (Lowin *et al.*, 1994).

Killer lymphocytes need some innate mechanism of protection against perforin-mediated cytolysis. Liu and co-workers (1995), describe protein present on the surface of cytotoxic lymphocytes, called 'protectin', which may protect these cells from perforin-mediated cytolysis. These authors found however, that some killer lymphocytes lacking protectin were still resistant to perforin-mediated cytolysis. It is possible that these cells undergo membrane repair, or that some other intracellular mechanism (as yet unidentified) is playing a role (Liu *et al.*, 1995).

Results of studies undertaken indicate conclusively that perforin is involved in lymphocyte-mediated cytolysis *in vivo* (Liu *et al.*, 1995).

Radoševic *et al.*, (1995-a) have shown that NK cells may penetrate into the cytoplasm of the target cell, a phenomenon entitled in-conjugation, when the killer cell has penetrated completely or partly into the target cell. When the entire cell has penetrated into the target cell it is called emperipolesis. This phenomenon has also been observed *in vivo* on biopsy samples (Garcia-Peñarrubia *et al.*, 1992).

An increase in F-actin has been found in conjugated NK cells in particular at the site of conjugation with target K562 cells. A similar increase in F-actin concentration was found in the conjugated K562 cells, coinciding with the appearance of filamentous actin structures, occurring at the point of contact. These alterations occurred after the increase in F-actin content of the NK cells, which was accompanied by increasing cytotoxic activity. The results observed were probably due to the interaction between activated NK cell and K562 target cell. The changes of F-actin organisation could be either as a result of cell damage caused by the NK cells, or a strengthening of the cell membrane in an attempt to resist the attack (Radoševic *et al.*, 1994).

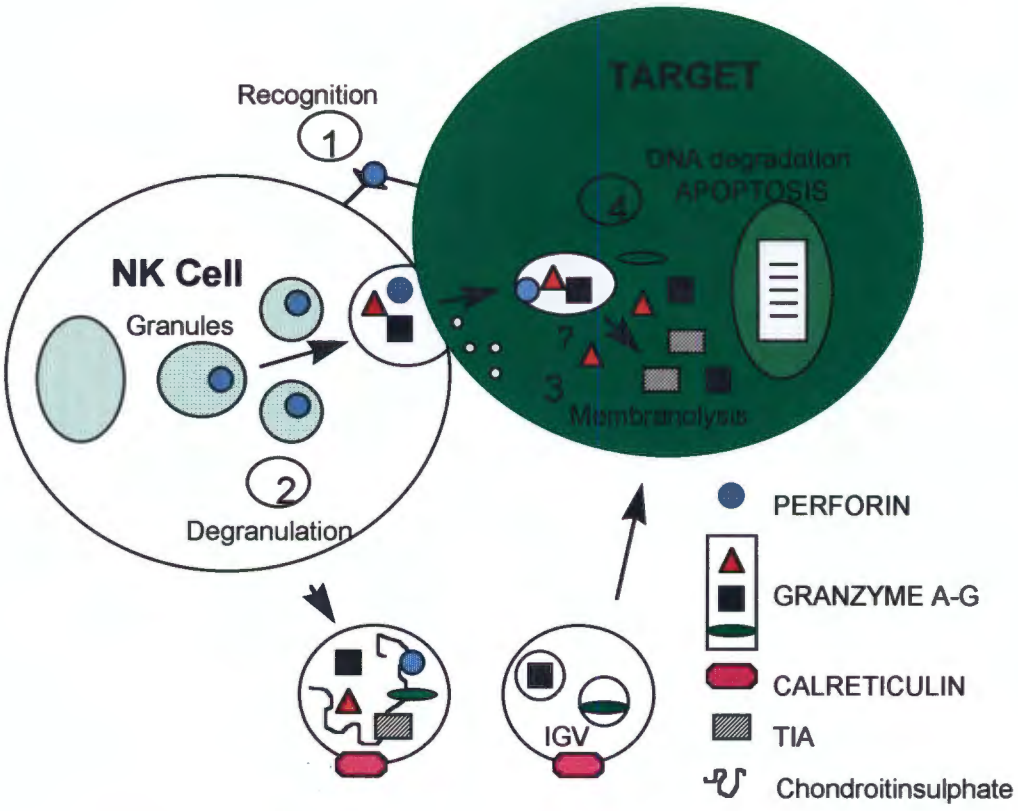


FIGURE 4. A diagrammatic representation of the perforin/granule exocytosis model of cytotoxicity.

1. Recognition and target-effector cell conjugation.
2. Cytoplasmic granule contents containing perforin, granzymes, calreticulin, TIA (a RNA-binding protein) and proteoglycans, are exocytosed into the intercellular space.
3. Perforin binds to target cell membrane and forms transmembrane lesions.
4. Access of granzymes and calreticulin into cytoplasm of target cell inducing apoptosis. The exocytosed proteins or intragranular vesicles (IGV) are possibly endocytosed and then translocated into the cytoplasm (Lowin *et al.*, 1994).

1.2.10. MEASUREMENT OF NK ACTIVITY

Natural killer activity (NKA) can be measured as percentage cytotoxicity, using a 4 hour chromium release assay, which is the most widely used assay to quantify this (for more detail as to methodology, see chapter on Materials and Methods).

In normal healthy individuals NKA does not appear to correlate with the number of circulating NK cells. As a result of this, it is necessary to perform NKA assays as well as enumeration of NK cells, when evaluating patients for defects in natural immunity (Whiteside, 1991).

It has been found that relatively pure populations of CD16 +ve cells lyse K562 targets efficiently without the need of any accessory cells (Reynolds and Wiltrout, 1989).

It is important to use fresh NK cells rather than cryopreserved cells, as cryopreservation sometimes decreases NKA. It is also imperative that medium contains foetal calf serum

rather than human serum, as human immunoglobulin has been shown to inhibit NKA (Whiteside *et al.*, 1990).

1.2.11. FACTORS AFFECTING NKA

NKA appears to remain relatively stable over time, although there may be a degree of biologic variability in the same individuals measured over time. It is thus recommended that serial monitoring of NKA is undertaken to detect outliers which may occur. The variability in NKA in normal controls has been shown to be 15% above and below the mean for that person. Only changes in patients NKA above this variable change can be considered as significant. NKA demonstrates circadian as well as circannual rhythms and it is important to bleed patients at the same time of day, usually the morning or early afternoon as these are times when NKA is maximal (Whiteside, 1991; Whiteside and Herberman, 1989; Urba *et al.*, 1987).

Illness e.g. infection, continued stress, or drugs e.g. corticosteroids, hormones, biological response modifiers or chemotherapy will alter NKA. NKA is also influenced by age, sex and exercise (Whiteside, 1991; Whiteside and Herberman, 1989; Kurago *et al.*, 1995; Whiteside *et al.*, 1990). NKA is increased with exercise, although overtraining causes suppression of immune function (Shepard and Shek, 1995).

1.2.12. NK CELLS AND T CELLS

The functional and phenotypic differences between B cells and NK cells are apparent, but the distinction between CTL's and NK cells has been the cause of much controversy. Both NK cells and T cells display phenotypic and functional similarities (Lanier *et al.*, 1986). CD56 has also been found on a small portion of T cells, but CD16, is not typically present on these cells. By using dual markers, it is possible to distinguish between these two populations. NK cells are CD3- CD56+, whereas T cells are CD3+ CD56+ (Lotzová, 1991).

To date no cell surface markers have been found definitively distinguishing T and NK cell progenitors (Spits *et al.*, 1995). Both NK and T cells that have been activated with IL-2 are able to kill tumour cells independent of MHC. These MHC-nonrestricted (MHC-NR) CTL display similar LGL morphology to NK cells and they are able to lyse K562. MHC-NR restricted CTL account for 1-2% of PBMC's and have less cytolytic activity than NK cells (Lanier *et al.*, 1986). There are, however, distinct differences between these two populations. T cells are thymus dependant for both differentiation and function, while NK cells are thymus-independant as NK cells are fully functional in athymic mice (Lotzová *et al.*,

1978). NK cells have the ability to kill tumour cells without prior activation, but MHC-NR T cells cannot (Lotzová, 1991).

NK cells and T cells complement each other in their surveillance against tumour cells. Target cells that escape T cell recognition due to lack of or suboptimal levels of class I antigens (often a result of tumourigenesis), become susceptible to NK cells (see the following section on NK cells and MHC class I) (Moretta *et al.*, 1994-b; Kos and Engleman, 1996).

1.2.13. NK CELLS AND MHC CLASS I

As NK cells are able to lyse cells that express allogeneic MHC antigen and also cells lacking MHC, the activity has been referred to as non-MHC-restricted. Progress has been made in understanding why NK cells may lyse one target and fail to lyse another target cell. An inverse correlation was found between MHC class I expression on target cells and their susceptibility to NK-mediated cytotoxicity. Either a lack of expression of MHC molecules, or a masking of these molecules would render these cells sensitive to NK cytolysis, which was proposed by Kärre and co-workers in the "missing self" hypothesis. NK cells therefore play a vital role in eliminating cells that do not express MHC class I molecules. Target cells become susceptible to NK cytotoxicity if expression of one or more MHC class I alleles are defective (Moretta *et al.*, 1996; Moretta *et al.*, 1997). Colonna (1996), states that NK cells have the ability to recognise the loss of even a single class I allotype and not necessarily all class I molecules. Target cell lysis by NK cells appears to be regulated by specific activating signals (positive signals) that initiate cytolytic response and inhibitory receptors (negative signals) that down-regulate cytolysis. There is presently a limited understanding in the processes of molecules responsible for positive signalling in NK cells, but it is probable that the interaction of different receptors occurs, (e.g. CD2, CD16 and CD69) depending on the activation state of the NK cell. These receptors have been termed killer-cell activatory receptors (KAR) or natural killer cell activatory receptors (NKAR), with a molecular mass of 50 kDa. The involvement of receptors in negative signalling is well defined - the presence of MHC class I on target cells prevent NK cell-mediated cytotoxicity. NK cells express receptors for polymorphic MHC class I molecules that inhibit killing of target cell bearing appropriate alleles. These receptors have been termed killer-cell inhibitory receptors (KIR) or natural killer cell inhibitory receptors (NKIR) with a molecular mass of 58 kDa. The inhibitory receptors prevent the killing of normal cells and focus cytolytic activity towards cells with reduced MHC class I. Presently more is known about the prevention of NK activation than what causes this activation. KIR's are characterised by the ability to interact with a large panel of MHC class I allele products (termed promiscuous recognition). KIR's have the ability to transduce a negative signal which causes inhibition of natural cytotoxicity as well as ADCC. KIR's serve as receptors for MHC class I molecules and transduce inhibitory signals

in NK cells when engagement with their ligands takes place. KAR's expressed on NK cells are always co-expressed with at least one KIR. Furthermore, KIR inhibitory functions always predominate over KAR triggering of cell lysis. This may be important in the prevention of autologous cells from lysis (Yokoyama and Seaman, 1993; Kurago *et al.*, 1995; Raulet and Held, 1995; Lanier and Phillips, 1996; Spits *et al.*, 1995; Lopez-Botet *et al.*, 1996; Yokoyama 1995; Colonna, 1996; Peña and Solana, 1992; Kägi *et al.*, 1995; Raulet, 1996; Warren, 1996; Long *et al.*, 1997; Long and Wagtmann, 1997; Renard *et al.*, 1997; Cantoni and Biassoni, 1997; Cambiaggi *et al.*, 1997). In summary therefore, NK cells express both activating and inhibitory receptors. Engagement of the activating receptor (the lysis receptor) results in either granule exocytosis and/or activation of the Fas/FasL pathway causing destruction of the target cell. Presently the identity of the lysis receptor remains uncertain. Engagement of the inhibitory receptor by class I MHC molecules on the target cell abrogates this lytic process (Reyburn *et al.*, 1997.)

The presence of MHC class I on target cells affects T cells and NK cells in opposite ways, although they use it to distinguish self from non-self. NK cells are not MHC restricted i.e. they are inhibited by self MHC class I, therefore they will kill cells lacking MHC class I. The presence of MHC class I on target cells inhibits killing by NK cells, i.e. NK cells kill all cells not recognised as self. T cells are MHC restricted i.e. they only kill targets expressing self MHC but containing foreign peptides in the groove. T cell recognition of target cell MHC allows killing, but NK recognition of target cell MHC prevents the killing by NK cells. In the recognition of "missing self", NK cells complement the cytotoxic response mediated by CTL. Due to the fact that some virus-infected cells or tumour cells have a down-regulation of the expression of MHC class I molecules, they would escape the CTL-mediated lysis. NK cells would be important in the elimination of these MHC-negative cells (Yokoyama and Seaman, 1993; Kurago *et al.*, 1995; Raulet and Held, 1995; Lanier and Phillips, 1996; Spits *et al.*, 1995; Lopez-Botet *et al.*, 1996; Yokoyama 1995; Colonna, 1996; Peña and Solana, 1992; Kägi *et al.*, 1995; Raulet, 1996; Moretta *et al.*, 1996).

Lobo and Patel (1994) have found that MHC class II molecules also serve a protective role for some B-lymphoid tumour cells against both NK and LAK - mediated cytolysis.

Mixed lymphocyte reaction activated NK cells (CD3 depleted) can lyse PHA induced blasts from normal allogeneic donors, but not autologous donors. (Moretta *et al.*, 1994-a; Ciccone *et al.*, 1992).

K562, which is MHC class I negative is highly susceptible to NK cells, but it was found that on induction of class I expression, these cells became NK resistant (Gronberg *et al.*, 1988). The above holds true for autologous cells, when MHC class I molecules are lost or masked,

they become NK-sensitive (Moretta *et al.*, 1994-b). Two theories have been proposed to account for this phenomenon (1) The effector inhibition hypothesis: presence of MHC class I on target cells sends a negative signal to NK cells preventing cytotoxicity, (the presence of MHC class I molecules may form the protective element against NK lysis (Moretta *et al.*, 1994-b)) or (2) The target interference hypothesis: MHC class I antigens on target cells mask the recognition by NK cells of NK target structures. Evidence appears to favour the former theory (Yokoyama and Seaman, 1993; Trinchieri, 1994; Kurago *et al.*, 1995).

Some target cells expressing MHC class I antigens are susceptible to NK lysis, but it is possible that the tumour cells may lack expression of single class I alleles which are functioning as protective elements (Versteeg *et al.*, 1989). By using allele-specific reagents, it was found that the loss of a single allele appears to be relatively common in some tumours (Moretta *et al.*, 1994-b).

It is possible that NK cells are advanced in their recognition of targets to enable them to distinguish self from non-self, NK-sensitive targets and different targets for LAK cells (Lanier and Phillips, 1992).

1.2.14. NK CELL ACTIVATION AND CYTOKINE PRODUCTION

Much of the evidence for cytokine production by NK cells has been obtained from *in vitro* experiments and it is difficult to directly compare this to an *in vivo* situation. Many experiments are unreliable, due to the presence of contaminating cells in NK enriched populations. Bearing this limitation in mind, it is clear that NK cells are capable of producing cytokines (Perussia, 1991; Naume and Espevik, 1994). Production of cytokines by NK cells possibly forms one of their essential biological functions. Cytokines are important in self-renewal, activation, cytotoxic function and in regulation of haemopoietic and lymphoid compartments (Lotzová, 1993). Activation of NK cells in response to various stimulatory factors, causes secretion of IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-12, IFN-γ, granulocyte/macrophage colony-stimulating factor (GM-CSF) and TNF alpha and beta and chemotactic factors (Trinchieri, 1994; Lotzová, 1993; Perussia, 1991; Naume and Espevik, 1994). Results of different studies have been contradictory as to the exact profile as given above, but this is probably due to the reasons mentioned previously. Studies appear to agree that resting NK cells are unable to secrete IL-2. TNF is secreted by NK cells in response to IL-2 and both TNF-α and TNF-β can be detected in the supernatants. This endogenous TNF production appears to be important in LAK activity and NK proliferation. Neutralising antibodies to TNF inhibit the IL-2 induced cytotoxicity and proliferation (Naume and Espevik, 1994). Due to the fact that NK cells produce γ-IFN, TNF and GM-CSF, it is clear that NK cells cause either negative or positive regulatory signals for haemopoiesis. NK

cells are thus able to modulate the activities of other leukocytes or leukocyte precursors (Naume and Espevik, 1994). Different cytokines have unique effects on NK cells, thus NK cells *in vivo* are able to affect immune reactions and haemopoiesis differently depending on the cytokine profile present (Naume and Espevik, 1994).

NK cells enriched from the peripheral blood were found to lyse freshly explanted autologous and allogeneic tumour cells as well as K562 (a NK sensitive cell line) and to release a functionally defined lymphokine called LGL-derived cytotoxic factor. LGL-derived cytotoxic factor was produced as a result of co-culturing PBMC with autologous tumour cells. Other cytokines may lyse tumour cell lines, but this factor is novel in that it has been found to lyse fresh human tumour cells. This factor was also found to spare nonmalignant cells (Uchida and Fukata, 1993).

1.2.15. NK CELL RESPONSE TO CYTOKINES

IL-2 is a cytokine with potent stimulatory properties and was the first immunological agent which demonstrated an anti-tumour effect by activating immune effectors. IL-2 also causes lymphoid proliferation (both *in vitro* and *in vivo)*, cytokine production, increase in the cytotoxic granule content, enhancement of adhesion molecule expression and increased cytotoxic activity (Tartour *et al.*, 1992; Naume and Espevik, 1994; Lotzová, 1993; Foon, 1989). NK cells cultured with IL-2 *in vitro* i.e. LAK cells show enhanced cytolytic capabilities and increased their antitumour activity to both NK-susceptible as well as NK-resistant tumour target cells *in vitro.* LAK effectors generated from CD3- cells are usually found to be positive for CD16 and CD56 markers. LAK cells are able to kill their targets in an MHC non-restricted manner i.e. LAK cells, have the ability to kill tumour cells expressing MHC class I or MHC negative cells (Grimm *et al.*, 1982; Hercend *et al.*, 1983; Perussia, 1991; Lanier and Phillips, 1992; Pross and Lotzová, 1993). LAK cells can be regarded as a function rather than a cell type or lineage (Foon, 1989; Shau *et al.*, 1993; Lotzová, 1993). LAK cells are able to lyse a greater selection of freshly explanted tumour cells than unactivated NK cells (Moretta *et al.,* 1994-b; Perussia, 1991). LAK cells have shown cytolytic activity towards autologous tumour cells in a short-term ^{51} Cr release assay (Reynolds and Wiltrout, 1989). The enhanced cytotoxicity of LAK cells is not due to an increase in the perforin content, but is probably due to increased expansion of adhesion molecules, allowing easier attachment to target cells. Two subsets of CD56+ cells have been stained, CD56 bright and dim. When these subsets are incubated *in vitro* with IL-2, it was found that the CD56 bright subset was stimulated with up to 100 times lower doses of IL-2 than the dim set. At the completion of the 7 day incubation period it was found that the bright population proliferated faster and 97% of the population consisted of this subset. This phenomenon is possibly due to the fact that CD56 bright cells express more of the high affinity IL-2R and its beta chain (Konjevic *et al.,* 1995).

In a study conducted by Caldera and co-workers (1992), LAK cells generated from normal controls and NHL patients were found to produce comparable levels of cytotoxicity. No correlation was found between the stage of lymphoma and the *in vitro* response of NK cells to IL-2 (Caldera *et al.*, 1992).

Zamkoff and co-authors (1990) conducted a study using PBMC samples from patients with relapsed or unresponsive NHL to see whether it was possible to generate *in vitro* LAK activity by using the NK resistant but LAK sensitive cell line, Daudi. All patients were able to generate LAK activity. In one third of these patients LAK activity was found to be less than normal controls and two thirds of them had LAK activity greater than or equal to that of the normal controls. There was no correlation of LAK activity to either histological subtype or amount of prior chemotherapy. The amount of LAK activity generated by either patient or normal control tended to remain stable over time.

NK cells are usually described as being nonadherent. This is only true for resting NK cells, but not for activated NK cells. NK cells that have been activated by IL-2 can adhere to plastic and have been termed adherent LAK cells (Lotzová, 1993).

IL-6 enhances NK, but not LAK activity, whereas IL-7 has been found to cause high levels of LAK activity in 5 day NK purified cultures (Naume and Espevik, 1994; Spits *et al.*, 1995). TNF alone cannot influence NK activities, but when incubated with low concentrations of IL-2, it is found to cause proliferation, induce IL-2R alpha expression and LAK activity in NK cells (Naume and Espevik, 1994). Transforming growth factor-beta (TGF-β) inhibits NK functions. Incubation of IL-2 and anti-TGF-β was found to enhance LAK activity compared to IL-2 alone, thus signifying an endogenous regulatory role of TGF-β (Naume and Espevik, 1994).

1.2.16. NK CELL ACTIVATION BY α-IFN

NK cells that have been stimulated with α-IFN *in vitro* are found to have a greater spectrum of cytolytic activity against most tumours and possibly even some normal tissue (Lanier and Phillips, 1992; Lotzová *et al.*, 1993). α-IFN, however, does not cause NK cell proliferation (Naume and Espevik, 1994).

The enhanced cytotoxic activity of NK cells treated with α-IFN *in vitro* was not accompanied by a new recruitment of NK-target binder cells but by an increase in the frequency of killer cells in the conjugate fraction (i.e. nonlytic conjugate-forming cells or binders) (Lebow *et al.*, 1993; Jewett and Bonavida, 1995). NK cells produce α-IFN when they bind to target cells

and this could serve as a positive feedback mechanism regulating their lytic potential. The α-IFN can be detected by the use of anti-α-IFN antibody (Roitt *et al.,* 1987; Lebow *et al.*, 1993).

Jewett and Bonavida (1995) also observed that *in vitro* incubation of PBMC's with α-IFN resulted in a much higher cytotoxic function than NK enriched cells, suggesting a role of other cells in activation. Ozer and co-workers (1983) report an enhancement of 20-200% in NK assays *in vitro* following preincubation of PBMC with α-IFN at doses of 10-1000 international units/millilitre (IU/ml). These investigators found that there was no correlation between clinical response and *in vitro* enhancement of NKA. α-IFN causes similar augmentation of NKA in both normal controls and NHL patients (Mehta, *et al.*, 1989-a). Draca (1993) contradicts this by stating that in most patients with disseminated tumours, the NK cells cannot respond to α-IFN stimulation.

1.2.17. EFFECTS OF *IN VIVO* α-IFN TREATMENT ON IMMUNE FUNCTION

Patients' proliferative response to phytohaemagglutinin (PHA) and streptokinase-streptodornase (SK-SD) was profoundly suppressed at a high α-IFN dosage of 100 x 10^6 IU in a study conducted by Ozer and co-workers (1983). This suppression was found to return to the baseline levels after 1 month. The response to PHA was reported as being unaffected by other authors (Urba and Baseler, 1988). A depression in NKA was also observed at high α-IFN doses, with enhancement of NKA seen in only a few patients at low dosage. Neither of these results could be correlated with clinical responses. It has been noted that in cancer patients, the first α-IFN injection served to boost NKA, but subsequent treatment caused no response. Thereafter, continued frequent α-IFN treatment was found to often cause a decline in NKA (Reynolds and Wiltrout, 1989; Ozer *et al.*, 1983; Maluish *et al.*, 1983). NHL patients frequently exhibit depressed immunologic function and an explanation for those patients responding to α-IFN, could be that their cytotoxic function remained relatively intact. When α-IFN is used at high doses, the antiproliferative effects outweigh any immuno-enhancing effects (Ozer *et al.*, 1983).

It is possible that if a treatment regimen using α-IFN, which resulted in boosted NKA was used, a greater clinical benefit would be demonstrated for cancer patients (Maluish *et al.*, 1983). Whiteside and Herberman (1989) state that when high doses of α-IFN cause a depression of NKA, this is a form of toxicity and should be avoided, hence it is important that serial measurements of NKA are carried out to avoid this phenomenon (Ozer *et al.*, 1983). It is possible that a considerable difference exists between the maximum tolerated dose of α-IFN and the optimally effective dosage (Urba *et al.*, 1987).

Both T and B lymphocyte populations appeared to be unaffected by α-IFN treatment. The CD4+ and CD8+ populations, as well as the ratio between them, appeared almost unchanged at a range of α-IFN doses, which held true even if there was significant leukopenia. The relative monocyte counts were occasionally found to be elevated 24 hours after α-IFN administration (Ozer *et al.*, 1983). Urba and co-workers (1987) however, report an increase in CD8+ and HLA-DR+ cells and a decrease in CD4+ PBMC as result of α-IFN treatment. An increase in NKA was also reported, and the increase in these parameters correlated with the tumour response. These same authors report on another trial, by other researchers who found no alteration in either total lymphocyte counts or any subsets during α-IFN treatment but did find depression in NKA. Clearly there is no consensus of opinion. The different findings reported by the various authors could possibly be related to the use of either recombinant or non-recombinant α-IFN.

One of the major difficulties in evaluating α-IFN treatment is that by assessing the functions of cells present in the peripheral blood this does not truly reflect the immune status of a patient. Cells present in the blood are in constant motion and these cells may not be the ones requiring change (Urba *et al.*, 1987).

α-IFN affects the NKA of patients by vastly different amounts, even at the same dose and route of administration. This poses a problem as to correlation of changes at a particular dose or administration site (Urba *et al.*, 1987).

Serum levels during α-IFN treatment were measurable using a bioassay, indicating that α-IFN retains biologic activity *in vivo* (Ozer *et al.*, 1983). The measurement of serum α-IFN levels is however not a true reflection of a fully biologically active drug. The induction of the intracellular enzyme 2',5'-oligoadenylate synthetase in PBMC is recommended by some as a more sensitive indicator of α-IFN treatment. This enzyme is induced by α-IFN in a wide variety of cells. Others, however, have found that it does not correlate well with dosage and routes of administration (Urba and Baseler, 1988).

Problems arise in interpretation of immune changes resulting from α-IFN treatment, as some functions may appear enhanced, others depressed and others unaffected. For a meaningful comparison to be made, a large patient sample should be utilised to enable valid interpretation of resulting immune function (Urba and Baseler, 1988).

1.2.18. THE EFFECT OF IFN ON TARGET CELLS

If NK susceptible target cells are exposed *in vitro* to IFN (alpha, beta or gamma) prior to NK cell addition, the lysis of these target cells is often markedly diminished. IFN treatment neither affects the conjugate formation between the target cell and the NK cell, nor the susceptibility of the target cells to NK cytotoxic factor, released by the effector cells. IFN does however appear to reduce the capacity of target cells to induce activation of conjugated NK cells (Reiter, 1993).

α-IFN has antitumour activity, not only by stimulating cytolytic activity, but also by a direct action on the tumour cells (Shephard and Shek, 1995). Urba and co-authors (1987) found that the *in vitro* antiproliferative effects of α-IFN on the patients' own tumour was related to an *in vivo* tumour response.

1.3.0. IMMUNE DEFECTS IN NHL

Although NHL patients are generally accepted as having impaired humoral immunity, studies on B and T cell numbers in the peripheral blood has been conflicting. It appears that a drastic reduction in numbers of mature B-cells present in the peripheral blood is one of the important abnormalities in NHL (Janowska-Wieczorek *et al.*, 1987). Murray and co-workers (1980), report a reduction in absolute CD3+ cells as well as total lymphocytes in NHL patients with advanced disease (stage III and IV), when compared to NHL patients with localised disease (stage I and II). Lindemalm *et al.* (1983), found a reduction in absolute CD3+ and CD4+ cells in the peripheral blood. Absolute numbers of CD8+ cells were normal, giving a low CD4+ to CD8+ ratio. A relative reduction in CD4+ and CD8+ cells was also observed in the lymph node, but the ratio between these two subsets was comparable to normal patients. In a small study conducted by Balasem and Barker (1984) on untreated NHL patients, percentages of CD8+ cells was significantly increased and the CD4+ to CD8+ ratio decreased compared to normal controls. Büchi and co-workers (1984), found a reduction in absolute numbers of circulating CD3+ and CD4+ cells in low-grade NHL (according to the Kiel classification) and decreased CD3+, CD4+ and CD8+ cells in high-grade NHL patients. These same authors found normal numbers of circulating B cells. Bergmann *et al.* (1985), report a reduction of CD3+, CD4+ and CD8+ lymphocytes in NHL patients, which was further found to be stage dependent. The reduction in T cell subsets was found to be unrelated to histological grade, according to the Kiel classification. B lymphocyte numbers remained within normal limits at all disease stages. Previously treated NHL patients showed a more profound reduction of CD4+ cells causing a decline in the CD4+ to CD8+ ratio. CD8+ lymphocytes in previously treated NHL patients did not differ

significantly from other untreated patients or normal controls. In a study conducted by Janowska-Wieczorek *et al.* (1987) on NHL patients of various histological grades, a reduction in percentages of CD3+ and CD4+ cells, but not CD8+ cells was found. Burger and colleagues (1990) conducted studies on both high-grade and low-grade NHL patients. The number of CD3+, CD4+ and Leu-7+ and NK cells were found to be decreased in the lymph node, depending on the pathological state. This also held true for NKA. There was no change in the number of CD8+ lymphocytes. Datta and co-workers (1992) in their study of untreated NHL patients, found a significant decrease in absolute lymphocyte number, both T and B cell subsets, as well as the CD4+ subset. The low numbers of circulating B cells in the peripheral blood could be attributed to a block in B cell differentiation. These authors state that the functional abnormalities of blood lymphocytes in NHL appear to be related to the presence of active disease. The authors further postulate that this functional impairment may predict relapse in these patients.

Frydecka and co-authors (1992) found statistically significant depressed NKA in all NHL patients studied. This was also found to be true in the different categories of NHL, namely, low, intermediate and high-grade. This finding was markedly increased in patients in remission after either chemotherapy or radiotherapy treatment. The mean NKA reached normal limits in high-grade NHL and was found to be raised in intermediate and low-grade patients as compared to normal controls. Contradictory to this, Mehta and colleagues (1989-b) found NHL patients had depressed NKA prior to treatment which returned to normal following treatment. In another study, it was reported that NHL patients demonstrated NKA and NK cell number comparable to normal controls (Caldera *et al.*, 1992). Mehta and co-workers (1989-a) on the other hand reported depressed NKA in NHL patients at diagnosis which returned to normal limits after treatment. These authors found that the impaired function in these patients occurred after conjugation, which was enhanced after treatment. The apparent contradiction in results obtained could be related to the different histological types of NHL (i.e. low, intermediate or high grade), as many researchers did not limit their study to any particular grade.

Datta and co-workers (1992), report an impaired DNA synthesis following PHA stimulation in NHL patients. Noguchi *et al.* (1976), in a small study of only 16 patients, found a reduction in the PHA response at diagnosis, but normal percentages of T cells. King and co-workers (1976), found that both Hodgkin's disease and NHL patients (12 and 5 patients respectively) showed an impaired response to the mitogens PHA, pokeweed and concanavalin-A *in vitro,* as a result of radiotherapy or chemotherapy.

IL-10, IL-6, IL-2 and TNF-α are produced *in vivo* by NHL patients at the site of the tumour. The presence of IL-2 at the site of the tumour may be related to tumour-infiltrating cells rather

than being produced by tumour cells. Furthermore, in NHL patients, circulating IL-6, IL-10 and TNF-α have been found *in vivo* in serum and also to correlate with survival in patients with active NHL. IL-10, IL-6 ,IL-2 and TNF-α each stimulated proliferation of NHL tumour cells. A combination of all these cytokines caused the greatest level of proliferation of NHL tumour cells. These cytokines appear therefore to be important in promoting proliferation of tumour cells *in vivo* (Voorzanger *et al.*, 1996).

1.4.0. α-IFN PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM NHL PATIENTS

Ho and co-workers (1992) report a deficiency in the production of α-IFN by the PBMC's of low and high-grade NHL patients after stimulation with *Newcastle disease virus* and *Corynebacterium parvum*. When stimulated with PHA the IFN response was within the normal range, with the exception of LG-NHL. As *Newcastle disease virus* and *Corynebacterium parvum* induce mainly α-IFN, and PHA mainly IFN-γ, it would suggest that a deficiency exists in the α-IFN response of NHL patients.

1.5.0. NK CELLS AND PRIMARY TUMOUR CELLS

Most of the evidence on NK cell function in antitumour immunity has been gained from experimental animals. In mice it was found that NK cells were able to inhibit NK sensitive tumour cells *in vivo*. *In vivo* depletion of NK cells in experimental animals has resulted in depressed immunity against tumours (Kos and Engleman, 1996). Data also indicates that NK cell anti-tumour activity is highly expressed in the blood stream against a variety of tumour cells including some NK resistant tumours. This data highlights the importance of NK cells in the control of metastatic spread and the growth of primary tumours. NK cells have been found to accumulate at the sites of inflammation in both primary and transplanted tumours (Reynolds and Wiltrout, 1989). Kurago and co-workers (1995), report that NK cells are only able to control small numbers of injected tumour cells, whereas CTL are able to eliminate large numbers of immunogenic tumour cells. These cells therefore perform complementary functions to each other. Circumstantial evidence suggests a role of NK cells in the prevention of the initial development of tumours, as humans and experimental animals with depressed NKA have a higher incidence of tumours. Patients on immunosuppressive therapy who were found to have low NKA are at high risk of developing tumours, both lymphoproliferative and also carcinomas. An early and profound depression of NKA is related to tumour incidence, suggesting an interference with natural resistance mechanisms. All of the above confirms the immune surveillance theory - that tumour development is associated with, and in fact preceded by depressed immunity. Studies have been conducted with the bias that NKA is the major contributing mechanism. The possibility that this activity

is controlled by soluble factors *in vivo* must be borne in mind (Reynolds and Wiltrout, 1989). There appears to be a correlation between low NKA and incidence of familial cancers, therefore a genetic deficiency in NKA may be a predisposing factor to cancer (Strayer *et al.*, 1984). Draca (1993) states that there is clearly a correlation between clinical stage and progression of malignant disease and that in most patients with disseminated tumours, NK cells are not cytolytic and cannot respond to IFN stimulation. Evidence appears to be contradictory, with some researchers claiming low NKA to be a predisposing factor or a prognostic indicator in cancer, while other researchers feel that there is no evidence to prove that NK cells prevent the development of tumours (Klein and Mantovani, 1993). This is not surprising, given the wide range for NKA among normal donors. It remains unclear whether low NKA is as a result of the cancer or a contributing factor to the disease (Pross and Lotzová, 1993). Mehta and colleagues (1989-a) report that all NHL patients, irrespective of their histological type, showed significant depression in NKA and NK cytotoxic factor, when compared to normal controls. The ability of NK cells to recycle from one target to another, known as the maximal recycling capacity, was found to be depressed in some malignancies.

Freshly explanted tumour cells are usually found to be resistant to cytolysis by NK cells, however, when cultured they were found to become sensitive. This cytotoxicity could be mediated by CTL and not as a result of NK cells at all (Klein and Mantovani, 1993). It is therefore important to use fresh tumour cells to measure cytotoxicity (Uchida and Fukata, 1993). In cancer patients, it was found that NK enriched cell populations demonstrated a lower NKA than in normal controls, although the yield and number of NK cells was the same in both groups. This could possibly demonstrate a defect in killing activity in patients (Satam *et al.*, 1986).

In vitro resistant tumour cells (as measured by sodium chromate 51 (^{51}Cr) release assays) which are inoculated intravascularly into laboratory animals, then become susceptible to cytolysis by NK cells in the bloodstream. It is possible that *in vivo* destruction by NK cells is more efficient than that detected by the *in vitro* assay and other cells, or humoral factors may also play a role *in vivo* (Reynolds and Wiltrout, 1989). It seems probable, that the avoidance by tumour cells of NK cytotoxicity is only a part of a complex sequence of events prior to the formation of a primary tumour. NK cells possibly mediate their tumouricidal effects through soluble factors (Reynolds and Wiltrout, 1989).

Given the wide range of activities of NK cells, it appears that they may play an important role in host defence, particularly against abnormal cells early in their generation. Beige mice, with an NK defect, appear to be prone to the development of lymphoma, suggesting an important role of immune surveillance for NK cells. This NKA is non-specific (Krzanowski, 1991).

In experiments with perforin-deficient mice, it was found that perforin-dependent cytotoxicity of CTL's as well as NK cells are crucial in the control of tumour cell growth (Kägi *et al.*, 1995).

1.6.0. NK CELLS AND METASTATIC SPREAD

Investigations involv.ng experimental animals has contributed to the understanding of mechanisms involved in the intravascular killing of tumour cells. NK cells were found to play a vital role in the removal of tumour cells in the blood stream, thus preventing metastasis formation, although other factors such as neutrophils, monocytes, natural antibodies or complement cannot be excluded. Tumours may evade the NKA in the bloodstream by utilising the lymphatics for metastasising, as NK cell number is low in lymph nodes (Reynolds and Wiltrout, 1989). Augmentation of NKA could thus increase the rate of tumour cell elimination and metastasis formation, although data seems to indicate that stimulation of NKA is more effective for prevention rather than eradication of tumour metastases (Reynolds and Wiltrout, 1989).

It has been proposed that NK cells recognise and kill tumour cells during the blood-borne phase of the metastatic cascade, however when there is a large tumour burden NK cells are not as competent. This is possibly due to the fact that their lytic capacity is overwhelmed or depressed due to tumour release of prostaglandins (Shepard and Shek, 1995; Reynolds and Wiltrout, 1989).

NK cells are able to recognise and lyse a wide selection of tumour cells suggesting that these cells may be important in controlling proliferation and metastatic spread of heterogenous tumour cell types (Lotzová, 1993).

1.7.0. NK ACTIVITY AS A PROGNOSTIC ROLE IN CANCER

Evidence for NKA as a prognostic factor appears to be contradictory, with some researchers claiming cancer patients usually exhibit low NKA and others reporting that there was no difference between normal controls and patients. In a study involving NHL patients, NKA was found to be depressed, but this did not correlate with either stage or prognosis (Mehta *et al.*, 1989-a). Kedar and Klein, (1992), report that cancer patients do not usually demonstrate either impaired NK or LAK activity, nor does it correlate with clinical response. Lotzová (1991), on the other hand, feels that there is a correlation between low NKA and a higher incidence, progression or dissemination of cancer. This seems to be particularly true of the incidence of leukaemia (Lotzová, 1991). A genetic deficiency of NKA seems to be

associated with familial cancers (Strayer *et al.*, 1984). NKA is possibly important in identifying people at greater risk of cancer, particularly in the close relatives of cancer patients (Whiteside and Herberman, 1989). The NK cytotoxicity assay has been suggested as a tool for the identification of persons at a greater risk of development of cancer, or in the prognostic assessment of cancer patients to attempt to predict progression and dissemination so that early treatment can be instituted. Low NKA might be important in predicting poor response to treatment and shorter survival time. NKA could be important in the monitoring of immunotherapy (Whiteside and Herberman, 1989; Lotzová *et al.*, 1991; Lotzová, 1991; Whiteside *et al.*, 1990). Pross and Lotzová (1993), however, feel that as a result of the wide range of NKA in normal controls, the use of this assay in either identifying patients at risk of developing cancer, monitoring of treatment or as a prognostic indicator, appears to be very limited.

1.8.0. TUMOUR-INFILTRATING LYMPHOCYTES

Tumour-infiltrating lymphocytes (TIL) are lymphocytes found within the tumour. Freshly harvested TIL's seem functionally "inert" *in vitro* compared to normal T cells, by appearing relatively immuno-incompetent, unless activated by IL-2. It is possible that either the separation techniques, or the enzymatic treatment used to isolate TIL's might result in the functional impairment of these cells. TIL's often express activation antigens. TIL's are activated and expanded from the tumour, by the *in vitro* incubation of these lymphocytes in the presence of IL-2 (Tartour *et al.*, 1992; Lotzová, 1991; Kradin and Bhan,1993; Whiteside and Parmiani, 1994).

TIL's have been found to demonstrate good cytolytic activity after activation with IL-2, more so even than PBMC with LAK activity. TIL's were found to be 50 to 100 times more efficient at killing targets than LAK cells, and were found to be tumour specific (Rosenberg *et al.*, 1986; Duckett and Belldegrun, 1992).

Unstimulated NKA present in TIL's is low compared to the NKA of PBMC's. It was found that in NHL, NK cells were seldom present as TIL's (Pross and Lotzová, 1993).

In some cancers, TIL's were more difficult to culture than in others, thus it may not be possible to cultivate TIL's from all tumours. The observation was made, that TIL's may have harmful effects on normal bone marrow (Lotzová, 1991).

The number, type, activation state and memory status of T-cells present as TIL's in B-cell lymphoma has been suggested as predictors of clinical outcome (Grogan and Miller, 1993). In a study conducted by Strickler and co-workers in 1988, it was found that spontaneous

lymphoma regressions were more likely to occur in untreated LG-NHL patients with extensive helper T-cell infiltration into the lymph node. Untreated LG-NHL patients with progressive disease were found to have fewer T-cells as well as significantly less helper T-cells than patients demonstrating spontaneous regressions. The number of cytotoxic/suppressor T-cells and NK cells was found to be the same in both groups of patients. These findings were confirmed in a study conducted by Medeiros and co-workers in 1989.

Diaz and co-authors (1996), report on the presence of tumour infiltrating T lymphocytes in NHL. A progressive increase in both memory and total T cells from LG-NHL to intermediate grade NHL and high grade NHL and a correspondingly decreased naïve T cell population was noted. These authors recommend that studies be undertaken to determine whether *in vitro* expansion and adoptive transfer of the memory T cell subset would result in tumour cytolysis.

The aims of this current study were as follows:- (1) To identify patients at diagnosis who would respond to *in vivo* α-IFN treatment. (2) To predict at diagnosis patients' response to cyclophosphamide. (3) To predict relapse prior to the appearance of overt symptoms. (4) To identify a quantifiable level of immune status that is related to a decreased risk of relapse.

2. MATERIALS AND METHODS

2.1.0. PATIENTS

A total of 32 low grade (according to the Working Formulation) NHL patients were included in this study, (see Figures 6 to 10 for a breakdown of all the relevant parameters). All patients had histologically documented NHL from excised lymph node tissue not related to the human immunodeficiency virus. Prior to the commencement of medication, baseline blood samples were received. Patients then followed a course of chemotherapy, consisting of:- cyclophosphamide, oncovin (vincristine) and prednisone (COP) for a period of approximately 6 weeks and blood samples were again received and analysed (postcop). Patients who responded to treatment were then selected to either follow a course of oral cyclophosphamide or subcutaneous injections of α-IFN_{2b} (3×10^6 U) three times per week; Intron A, Schering-Plough (PTY) LTD, Isando, RSA), for two years, according to the availability of α-IFN treatment. Figure 5 summarises the treatment and indicates at which time points patients were bled. All patients selected for the trial, were informed and gave written consent. The research was approved by the ethics board. Blood samples were ideally received at three monthly intervals, but were not always received on schedule. Normal controls were run in parallel on every occasion. A total of 38 different normal controls were used, some of whom were utilised on more than one occasion.

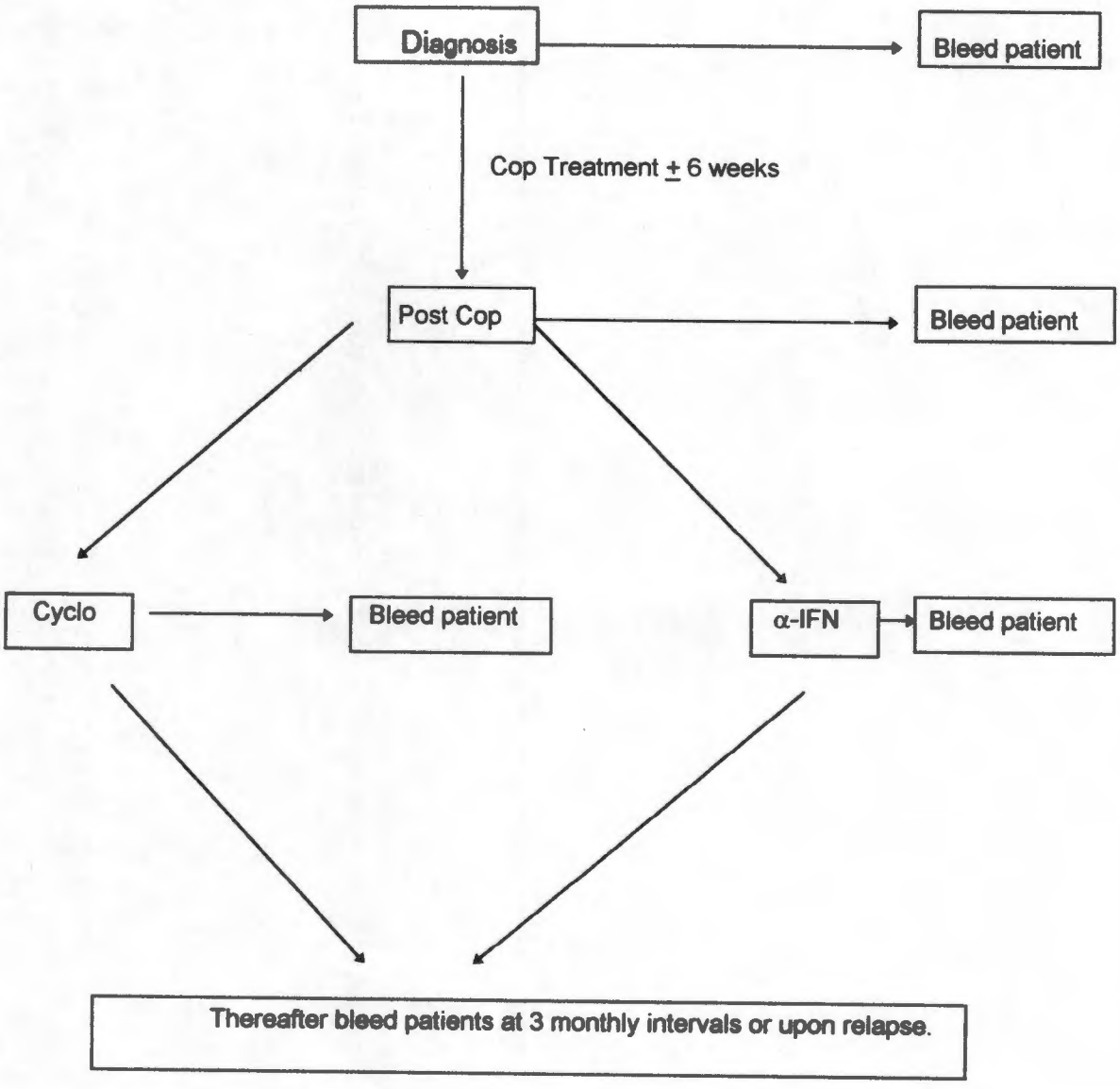


FIGURE 5. A diagrammatic representation of patient treatment and bleeding time points.

Cyclo = cyclophosphamide

COP = cyclophosphamide, oncovin (vincristine) and prednisone

2.2.0. PERIPHERAL BLOOD MONONUCLEAR CELL ISOLATION

PBMC were isolated as previously described (Ratcliffe *et al.*, 1994) - 50 ml of fresh venous blood from NHL patients, or 20 ml from normal controls was collected in heparinised tubes (Novo Nordisk (Pty) Ltd, Johannesburg, RSA; 5 U/ml blood) (cell recovery was suboptimal in patients due to treatment, thus requiring a larger volume of blood). The blood was centrifuged for 12 minutes at 300 x gravitational attraction (g) and the plasma removed immediately and stored at -20°C for cytokine analysis. The buffy layer was removed and diluted with an equal volume of phosphate buffered saline (PBS; Dulbecco 'A', Oxoid, Unipath Ltd, Hampshire, UK) and gently layered onto Ficoll-Hypaque (Lymphaprep -

Nycomed, Oslo, Norway) for PBMC isolation by density gradient separation. The gradient was then centrifuged for 30 minutes at 600 x g, the PBMC layer removed and washed 3 times by resuspending in PBS and centrifuging for 10 minutes at 200 x g. Cells were counted, using Turk's solution, (which stains the leukocytes, but lyses red blood cells) and then resuspended in tissue culture medium consisting of, Roswell Park Medical Institute media (RPMI 1640; Flow, ICN Biomedicals, Aurora, Ohio) containing 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate (Sigma Chemical Company, St Louis, USA), 20mM HEPES (Sigma Chemical Company, St. Louis, USA) supplemented with 10% heat inactivated pooled human AB serum for proliferation assays, or in 10% heat inactivated foetal calf serum (FCS; Delta bioproducts, Kempton Park, RSA) for both the NK and LAK assays.

2.3.0. PHENOTYPIC ANALYSIS

Flow cytometric analysis of PBMC was performed on an Epics Profile II (Coulter Electronics, Hialeah, Florida). Monoclonal antibodies used were CD3-FITC, CD4-RD1, CD8-RD1, NKH1-RD1 (anti-CD56) and when there appeared to be malignant B-cell spill-over into the blood, B4-FITC (anti-CD19) (Coulter Immunology, Hialeah, Florida). For determination of percentage NK cells present in peripheral blood, dual parameter flow cytometric analysis was performed using both CD3-FITC and NKH1-RD1 monoclonal antibodies. Similarly for determination of both CD4+ cells and CD8+ cells, dual parameter flow cytometric analysis was conducted using CD3-FITC and either CD4-RD1 or CD8-RD1. Non-specific antibody binding was evaluated by using the relevant mouse isotopic controls, to allow 2% false positives. Antibodies were used at concentrations suggested by the manufacturer. Lymphocytes were bitmapped to visually exclude monocytes and debris. Histogram data were generated for 10^4 events. In this way the percentage of cells positive for a particular surface marker was determined.

The full blood count and differential counts were determined by the Haematology Laboratory, Groote Schuur Hospital, enabling the determination of absolute lymphocytes and absolute leukocytes.

2.4.0. MITOGEN AND ANTIGEN PROLIFERATION

PBMC proliferation in response to mitogen and antigen was analysed in microculture as previously described (Ratcliffe *et al.*, 1994). PBMC obtained from both NHL patients and normal controls, were resuspended at 1×10^6/ml in tissue culture medium, supplemented with 10% AB human serum. Mitogenic stimulation was monitored in response to 5.75×10^{-3} mitogenic units/ml PHA (Wellcome Research Laboratories, Beckenham, UK). Antigenic stimulation in response to purified protein derivative of *Mycobacterium tuberculosis* (PPD;

Central Veterinary Laboratory, Weybridge, UK) at 3 μg/ml and SK-SD (Lederle Laboratory, Wayne, NJ; 250 U/ml streptokinase; 62.5 U/ml streptodornase) at a final dilution of 1:40 (following dialysis to remove preservatives), was also monitored. The SK-SD was heated at 56°C for 1 hour to inactivate the enzymatic activity, which was damaging to the cells, while retaining the antigenicity. These concentrations were found previously to elicit maximal thymidine uptake. PBMC were plated in triplicate in sterile 96-well u-bottom microtitre plates (Greiner GmbH, Frickenhausen, Germany) at 10^6 cells/ml and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 days (mitogens) or 6 days (antigens). For both day 3 and day 6 proliferation, triplicate wells were included, consisting of PBMC without mitogen or antigen for determining background (unstimulated) proliferation. Tritiated thymidine (Amersham International, Buckinghamshire, UK, specific activity 185 MBq/mmol) was added (2μci/well) on day 3 of mitogen stimulation and day 6 of antigen stimulation for a further 18 hours, to allow incorporation of the radiolabel into the proliferating cells. The cells were harvested onto titertek filter paper (Flow, ICN Biomedicals. Inc. Costa Mesa, CA), using an automated cell harvester (Titertek cell harvester, Flow Laboratories). The filter paper was air dried and the circular discs removed and placed into disposable plastic tubes (Zinsser Analytic, Frankfurt, Germany). Scintillation fluid was added (Insta-Gel II, Packard, Meriden, U.S.A.) and tritiated thymidine incorporation was measured in a beta counter (Tricarb liquid scintillation analyzer 1900CA, Packard) as counts per minute (cpm). Results are expressed as a stimulation index (SI), where SI = mean cpm's in stimulated wells/mean cpm's in unstimulated wells (background).

2.5.0. CYTOKINE ANALYSIS

2.5.1. PREPARATION OF SUPERNATANTS FOR CYTOKINE ANALYSIS

PBMC (3 x 10^6/ml/well) were cultured in tissue culture medium supplemented with 10% pooled human AB serum in 24 well tissue culture plates (Falcon, Becton Dickinson and company, Lincoln Park, New Jersey) and stimulated by PHA (5.75 x 10^{-3} mitogenic units/ml; Wellcome Research Laboratories, Beckenham, UK). The supernatants were collected after 24 hours and stored at - 20°C. If sufficient cells were isolated, an unstimulated well was also included, consisting of PBMC (3 x 10^6/ml/well) cultured in 10% pooled human AB serum in RPMI.

2.5.2. CYTOKINE ASSAYS

A commercial immunoradiometric assay (IL-2 IRMA, Medgenix Diagnostics, Fleurus, Belgium) was used to measure IL-2 concentration in the PBMC supernatants and plasma

samples according to the manufacturers' directions. A selection of patients from the various response groups and normal controls were analysed for IL-2.

2.6.0. MAINTENANCE OF TISSUE CULTURE CELL LINES

K562, the NK sensitive cell line, was established by Lozzio and Lozzio (1975) from the pleural effusion of a 53 year old female with chronic myelogenous leukemia in terminal blast crisis. This cell line has attained widespread use as a highly sensitive *in vitro* target for the NK assay (Lozzio and Lozzio, 1975).

Daudi, the NK resistant, but LAK sensitive cell line was derived from a 16 year old Black male with Burkitt's lymphoma by Klein and Klein in May 1967. This is a well characterized B lymphoblast cell line. Daudi should be handled as potentially biohazardous material under at least Biosafety Level 2 containment (Klein *et al.*, 1968).

The culture medium used for propagation of both K562 and Daudi cell lines, consisted of, RPMI (Flow, ICN Biomedicals, Aurora, Ohio) containing 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate (Sigma Chemical Company, St Louis, USA), 20mM HEPES (Sigma Chemical Company, St. Louis, USA) supplemented with 10% FCS (Delta bioproducts, Kempton Park, RSA). Cell lines were passaged and fresh medium added every 72 hours with cells resuspended at 1 x 10^5 cells/ml in 25 cm^2 tissue culture flasks (Nunc A/S, Inter Med, Roskilde, Denmark) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were routinely checked for Mycoplasma contamination, using an enzyme immunoassay Mycoplasma detection kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturers' directions. Cells were also routinely checked for purity using the Epics Profile II (Coulter Electronics, Hialeah, Florida) flow cytometer and a DNA propidium iodide stain (Coulter Immunology, Hialeah, Florida).

2.7.0. NATURAL KILLER ACTIVITY

Fresh NKA of PBMC from NHL patients and normal controls were evaluated in a standard 4 hour sodium chromate 51 (^{51}Cr) release cytotoxicity assay against the NK sensitive erythroleukaemic cell line K562, as previously described (Ratcliffe *et al.*, 1994). It is of utmost importance that target cells are utilised while in the log growth phase, to ensure high viability and good uniform uptake of the radioisotope (Whiteside *et al.*, 1990). K562 cells (1 x 10^6) were labelled with 250 µci ^{51}Cr (specific activity 370 MBq/mg, Amersham International, Buckinghamshire, UK) for 1 hour and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were then washed 3 times in cold PBS, supplemented with 10% FCS. Three thousand targets were added to each well of a 96-well u-bottom microtitre

plate (Greiner GmbH, Frickenhausen, Germany) in 100 µl 10% FCS in RPMI. PBMC were serially diluted in 10% FCS in RPMI to give final effector to target ratios of 90:1, 30:1, 10:1 and 3:1 in triplicate. It is imperative that medium contains FCS rather than human serum, as human IgG has been shown to inhibit NKA (Whiteside *et al*, 1990). Maximum ^{51}Cr release was determined by the addition of 100µl of 5% triton-X 100 (BDH Chemicals Ltd, Poole, England) to target cells alone. Spontaneous ^{51}Cr release was measured in the absence of effector cells. Plates were centrifuged at 50 x g for 5 minutes and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 hours. The assay was terminated by centrifugation at 200 x g for 10 minutes. From each well, 130µl of supernatant was removed and transferred to disposable counting tubes (Greiner, GmbH, Frickenhausen, Germany). Radioactivity of samples was measured in a gamma counter (Auto-gamma, scintillation spectrometer 5260, Packard). To avoid circadian variation all NK assays were performed at the same time of day. Percentage cytotoxicity was calculated as follows: (E-S)/(M-S) x 100, where E is mean cpm of experimental wells, S is the mean cpm for spontaneous release and M is the mean cpm for maximal release.

2.8.0. MICROSCOPIC ANALYSIS OF CYTOTOXICITY

A 4 hour cytotoxicity assay was carried out as described above without the addition of ^{51}Cr. At the termination of the 4 hour period and after the plate had been centrifuged at 200 x g for 10 minutes, 100 µl of supernatant was removed and 100 µl of Trypan blue added. The sample was well mixed and 100 µl subjected to cytospin centrifugation (Shandon Elliot, Shandon Southern Instruments Ltd., Camberley, Surrey, UK). The slide was air dried and counterstained with 0.5% carbol fuchsin. Slides were examined under a light microscope (Zeiss Axioskop 20, Carl Zeiss, Oberkochen, Germany) and photographed with a microscope camera (Zeiss MC80, Carl Zeiss, Oberkochen, Germany).

2.9.0. ALPHA INTERFERON ENHANCED NKA

α-IFN (Intron A, Schering-Plough (PTY) LTD, Isando, RSA - kindly provided by Schering-Plough (PTY) LTD) was prepared by resuspending in bacteriostatic water (Bactstat; Schering-Plough (PTY) LTD, Isando, RSA) at a concentration of 1 x 10^6 U/ml. This was aliquoted and immediately snap frozen, using liquid nitrogen. Immediately before use, aliquots were gradually thawed by placing in the refrigerator. Any unused α-IFN was discarded. α-IFN (1 x 10^4 U/ml concentration, as recommended by the suppliers) was incorporated into the NK assay.

2.10.0. LYMPHOKINE-ACTIVATED KILLER ASSAY

For measurement of LAK activity, as previously described, (Ratcliffe *et al.*, 1994), effector cells were generated by culturing 6-10 x 10^6 PBMC at 10^6/ml in 10% human AB serum in RPMI, supplemented with 100 U/ml recombinant human IL-2 (Cetus Corporation, Emeryville, CA - kindly provided by Dr Eli Kedar, Hadassah Medical School, Jerusalem, Israel) in 25 cm^2 tissue culture flasks (Nunc A/S, Inter Med, Roskilde, Denmark). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 7 days. LAK cells were pelleted at 200 x g and viable cells were counted using a vitally excluded stain, Trypan blue (0.02% aqueous solution), and resuspended in 10% FCS in RPMI, to give effector to target ratios of 90:1, 30:1, 10:1, 3:1, 1:1, 0.3:1, 0.1:1 and 0.03:1. Thereafter, the assay was performed in exactly the same way as for NKA described above, with the additional inclusion of the NK-resistant but LAK-sensitive Daudi cell line as a second target cell. Percentage cytotoxicity was calculated as previously described.

2.11.0. STATISTICAL ANALYSIS USED IN THE STUDY

The statistical program Sigma Plot (Jandel Scientific Corporation, USA) has been utilised in the compilation of the box and whisker graphs. A box and whisker plot is a graphical display that shows a measure of location (the median), a measure of dispersion (the interquartile range), and the presence of possible outliers and also gives an indication of the symmetry or skewness of the distribution. The box and whisker plot is made up of:- (1) Horizontal lines drawn at the median and at the upper and lower quartiles which are joined by vertical lines to produce the box; (2) A vertical line is drawn from both the upper and lower quartiles to the most extreme data points. In this way 90% of the data points are included. Short horizontal lines are added to mark the ends of these vertical lines; (3) Each data point beyond the ends of these vertical lines is marked with a dot (Rice, 1988).

The software program Statgraphics (STSC Inc., Rockville, MD) was utilised in the determination of p-values by the Mann-Whitney U-test and, where appropriate, the Wilcoxon signed-ranks test for paired data with p values of 0.05 or less being considered significant.

3. RESULTS

3.1.0. PATIENTS

3.1.1. EXCLUSION CRITERIA AND DIFFERENT TREATMENTS

Thirty two patients were available for evaluation, with some being excluded after the first few visits due to a change in treatment protocol. Patient samples should ideally have been received at three monthly intervals, but were not always received on schedule. Due to the fact that cell recovery was often suboptimal in patients, as a result of treatment, it was not always possible to perform all the tests required leading to incomplete data on some patients. The time points indicated in the figures are given as ranges, rather than definite points to overcome the above-mentioned difficulties. Ideally the patients in the two different treatment regimens should consist of equal numbers, but due to a delay in the supply of α-IFN, more patients were treated with cyclophosphamide than with α-IFN. Twelve patients were treated with cyclophosphamide and eight patients with α-IFN and twelve patients with regimens that differed from the protocol and have been referred to as "miscellaneous patients". The miscellaneous group of patients were included in the study until the treatment differed from the stipulated protocol. Normal controls were run in parallel on every occasion. A total of 38 different controls were used, with a large proportion being utilised on more than one occasion. On repeated testing of normal controls no significant inter-assay variation was found.

3.1.2.0. DISTRIBUTION OF NHL PATIENTS IN THIS STUDY

3.1.2.1. PATIENT CLASSIFICATION

Category C, according to the Working Formulation, accounted for the majority of patients in this trial (Figure 6).

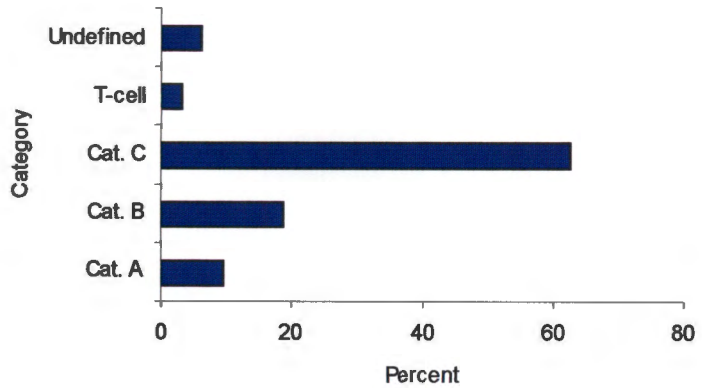


FIGURE 6. Patient Classification distribution.
An indication of the distribution of NHL patients according to the Working Formulation Classification.

3.1.2.2. PATIENT STAGING

The vast majority of patients in this trial were already at stage IV, according to the Ann Arbor staging method, when diagnosed (Figure 7). As previously mentioned in the Introduction, the majority of LG-NHL are at stage III or IV when detected, with only 15-20% being diagnosed at stages I and II. This trend was substantiated in the current study, although no patients were identified at Stage II.

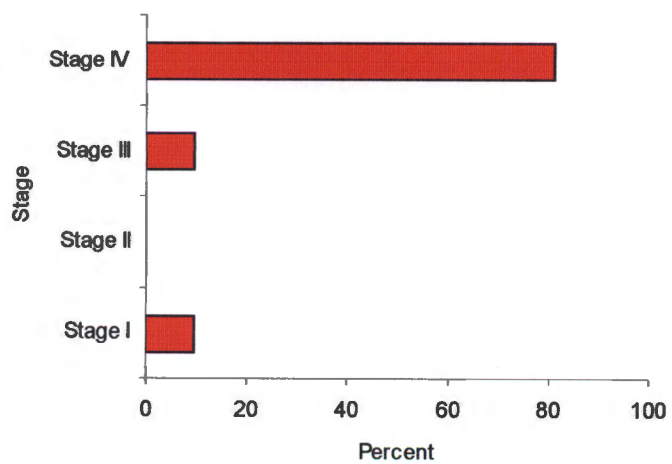


FIGURE 7. Patient stage distribution.
An indication of the Ann Arbor stage distribution of NHL patients.

3.1.2.3. PATIENT AGE DISTRIBUTION

The majority of patients in this trial were between 51-70 years of age (Figure 8). The finding that LG-NHL is more common in the elderly was confirmed in the current study. It was found that 22% of the patients were > 64 years of age; 72% were 35-64 years of age and 6% were < 35 years of age. This study was limited to adults which may result in the moderate discrepancy in percentages of patients found in the different age groups as discussed in the Introduction (Table 1).

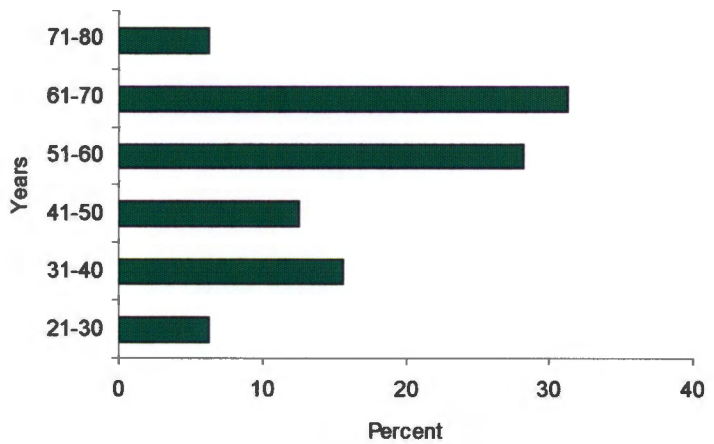


FIGURE 8. Patient age distribution.
An indication of the age distribution of NHL patients.

3.1.2.4. PATIENT SEX DISTRIBUTION

The male to female ratio of this trial was assessed and found to be 7:9 (Figure 9) which is different from that reported by Hancock (1995). Hancock was discussing all NHL's and not LG-NHL's in particular, which would explain this slight discrepancy.

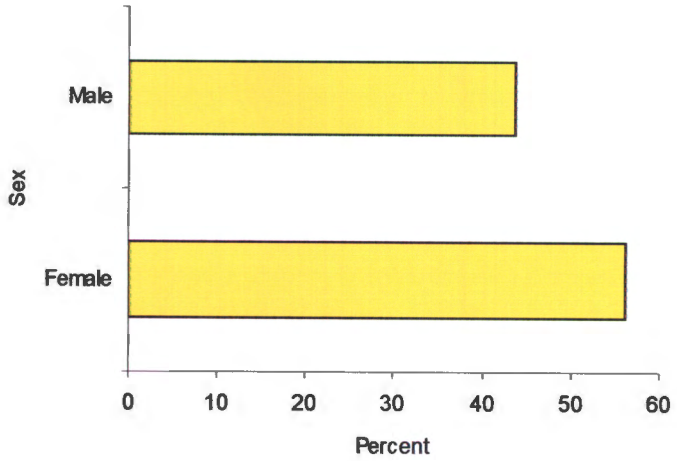


FIGURE 9. Patient sex distribution.
An indication of the sex distribution of NHL patients.

3.1.2.5. PATIENT RACE DISTRIBUTION

It was found that most patients in this study were white, with fewer coloured patients and only 1 black patient (Figure 10). This confirms the report by Greiner and co-workers (1995), where it was noted that the incidence of LG-NHL is low in blacks.

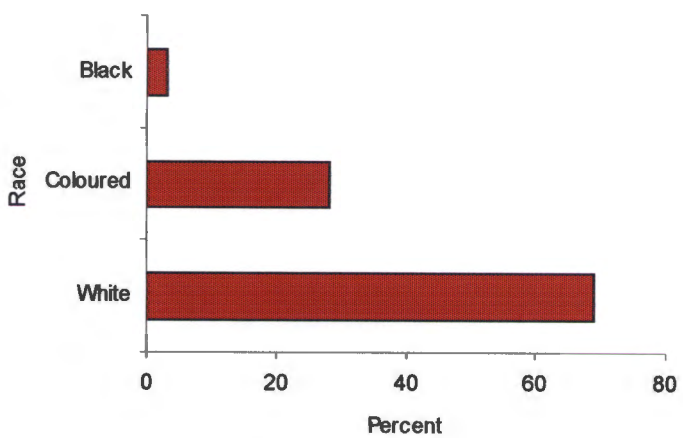


FIGURE 10. Patient race distribution.
An indication of the race distribution of NHL patients.

3.2.0. IMMUNE STUDIES PERFORMED ON PATIENT BLOOD SAMPLES

3.2.1. ANALYSIS OF NKA AND α-IFN ENHANCED NKA

Whiteside and co-authors (1990) recommend the use of lytic units in the estimation of NK cytotoxicity. In the determination of lytic units, the percentage cytotoxicity at all the effector to target ratios should fall in the linear portion of the graph. In this study however all the effector to target ratio cytotoxicity values did not satisfy this requirement. The use of lytic units did not accurately reflect the cytolytic potential of the cells and was not suitable in this study for comparative purposes. It was decided therefore to rather compare percentage cytotoxicity at a specific effector to target ratio, which was more representative of the results.

An indication of NKA is shown as a box and whisker plot (Figure 11-A). On the Y-axis the percentage cytotoxicity is shown at the effector to target ratio of 10:1. It should be noted that all other effector to target ratios gave similar trends. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop (after completion of COP treatment) and at various intervals until >100 weeks after the commencement of treatment. The mean of NKA for controls (18.43 ± 10.11) (mean ± standard deviation) was found to be lower than that of patients at diagnosis (24.57 ± 15.94), although this difference was not statistically significant. The NKA was found to decrease after the commencement of treatment. The mean for postcop was (17.76 ± 10.12) and this diminished even further until it reached the lowest level at 51-70 weeks (11.5 ± 9.32). At 71-100 weeks it increased slightly (12.39 ± 5.57) until >100 weeks (16.03 ± 10.61). It must be borne in mind that the median rather than the mean is indicated in box and whisker plots. The difference in this cytotoxicity between patients and controls was found to be statistically significant at 30-50 weeks with a p value of <0.05. The difference between patients' baseline results and 30-50 weeks ($p<0.05$) and 51-70 weeks ($p<0.05$) was found to be statistically significant. In all the findings that are found to be statistically significant, sample size has been taken into account, but it must be pointed out that with time, the sample size in all assays undertaken was decreased.

Results of studies investigating the NKA of NHL patients have been contradictory. Frydecka and co-authors (1992), found statistically significant depressed NKA in all NHL patients studied, as well as in the different categories of: low, intermediate and high-grade. Contrary to this, Mehta and colleagues (1989-b), found NHL patients had depressed NKA prior to treatment which returned to normal following therapy. In another study, it was reported that NHL patients demonstrated NKA and NK cell numbers comparable to normal controls

(Caldera *et al.*, 1992). The apparent contradiction in results obtained could be related to the different histological types of NHL i.e. low, intermediate or high grade.

The reason for the finding of a decrease in NKA until 51-70 weeks and then an increase is probably due to chemotherapy. It has been established that NKA is diminished in patients treated with chemotherapy (Whiteside *et al.*, 1990). This finding is confirmed in the current study.

α-IFN augmented NKA (NKA + α-IFN) using 1 x 10^4 U/ml is shown in Figure 11-B. A very similar trend in results with α-IFN was detected as that obtained in the NKA results (Figure 11-A). The patients' baseline NKA + α-IFN (33.89 ± 18.68) was found to be higher than that of the normal controls (26.89 ± 10.08). It is a well established fact that α-IFN enhances the cytolysis of mature PBMC derived NK cells *in vitro* (Kedar and Klein, 1992; Pollack, 1993). This was confirmed in the present study. Controversy exists as to the possible enhancement of patients' NKA after *in vitro* α-IFN incubation. Mehta and co-workers (1989-a) report similar augmentation in both NHL patients and controls. Once again the study by Mehta and co-workers was not limited to LG-NHL, hence the possible slight discrepancy. Draca (1993) contradicts this by stating that in most patients with disseminated tumours, the NK cells cannot respond to α-IFN stimulation.

Although the postcop results (33.02 ± 12.12) were similar to baseline, NKA + α-IFN was depressed at 30-50 weeks (18.25 ± 16.49), which is probably an indication of the effects of chemotherapy. Thereafter the activity rose steadily, until 71-100 weeks (27.32 ± 7.03) at which point the mean of the patients' NKA + α-IFN were similar to controls (26.89 ± 10.08). The difference between the controls and patients NKA + α-IFN results was found to be statistically significant after 30-50 weeks, with $p<0.01$. Patients' baseline and 30-50 week results were also statistically significantly different with $p=0.01$, as was the difference between postcop and 30-50 weeks with $p<0.05$. The majority of both patients and controls were found to have enhanced NKA after *in vitro* α-IFN incubation.

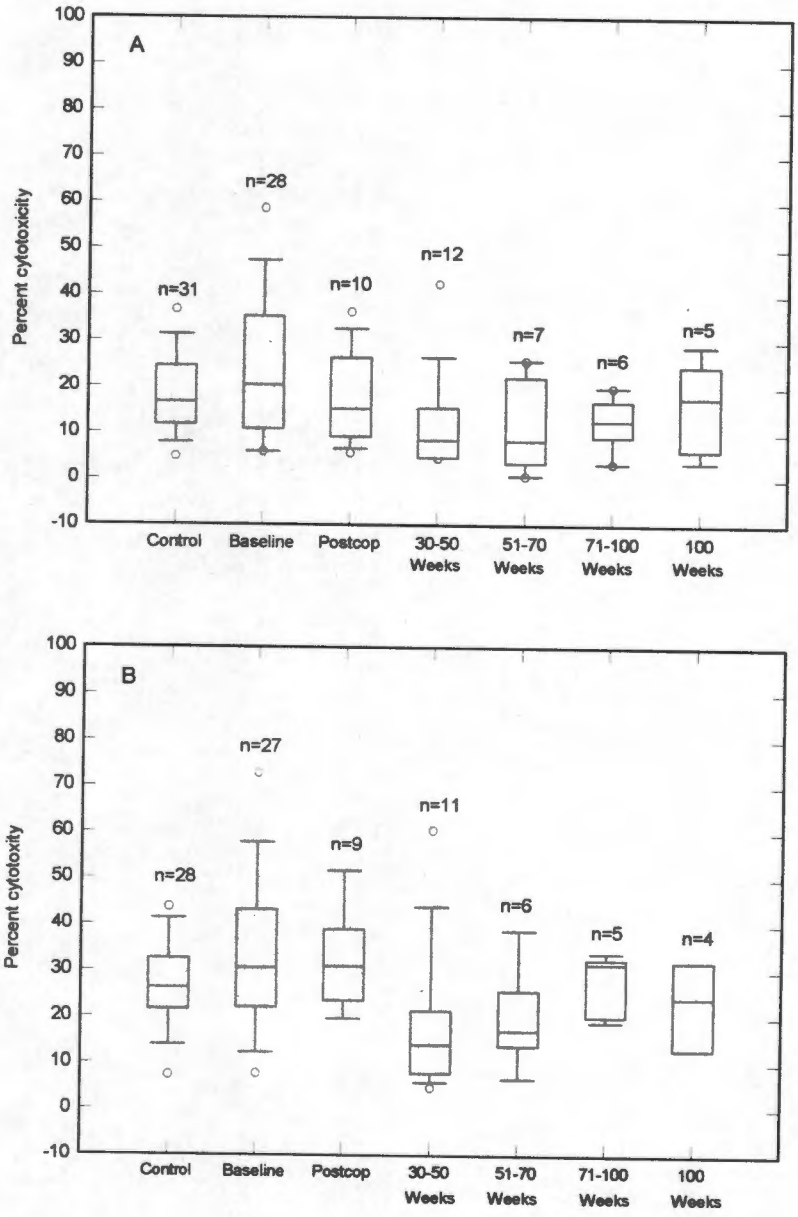


FIGURE 11. NKA and α-IFN enhanced NKA in controls and patients over time.
NKA (A) and α-IFN enhanced NKA of PBMC (B) from LG-NHL patients was evaluated in a standard 4 hour ^{51} chromium release assay against K562. α-IFN (1 X 10^4 U/ml) was incorporated into the NK assay (B). On the Y-axis percentage cytotoxicity can be seen at an effector to target ratio of 10 to 1. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until >100 weeks after the commencement of treatment.

3.2.2. INDIRECT CALCULATION OF NK CELLS REQUIRED TO KILL ONE K562 TARGET CELL

By utilising the phenotypic analysis in determining the percentage of NK cells (CD56+) present in the peripheral blood, and with the addition of a known number of lymphocytes in the NK assay, it was possible to determine the number of NK cells present. The number of K562 cells added was also known, therefore it was possible to determine the number of NK cells that were required to kill 1 K562 target cell (Figure 12-A), and similarly, after boosting with α-IFN (Figure 12-B). The number of patients in this graph is different to that in Figure 11 A and B, due to the fact that not all patients had both NKA and percentage NK cells assayed on every sample submitted, as a result of insufficient cell recovery. It was found that the patients' baseline results indicated that more NK cells were required to kill 1 target K562 cell (10.76 ± 8.2) than in normal controls (8.28 ± 4.94) (Figure 12-A), although this difference did not reach statistical significance. Fewer NK cells were required to kill a K562 target cell at postcop (7.98 ± 3.46), but by 30-50 weeks (15.66 ± 6.27), this number had increased dramatically, the difference being statistically significant with $p<0.05$. This finding is possibly as a result of the diminished NKA or alterations in distribution of NK subsets, due to chemotherapy. The difference between the NK cell number required to kill 1 target cell in controls and patients at 30-50 weeks is significant with $p<0.01$.

NKA was enhanced by α-IFN (Figure 12-B) and the trend of these results resembles those in Figure 12-A, except that the difference between controls (5.16 ± 4.02) and patients at baseline (6.77 ± 4.82) does not appear to be as marked. Initially at postcop (5.16 ± 2.49), there appears to be some improvement in that fewer NK cells boosted by α-IFN are required to kill the 1 K562 target cell. By 30-50 weeks (12.99 ± 5.62) however, the number of cells required has increased significantly between baseline values ($p<0.05$) and controls ($p<0.01$). This is once again thought to be as a result of chemotherapy.

Several factors would limit the confidence with which one can interpret the results obtained, using this indirect calculation of lytic activity. These include the heterogeneity of NK subsets, and the use here of only one NK cell marker (CD56). Afify and co-workers (1990), reported that CD56+ cells mediate the most NKA in the peripheral blood. However, LeFever and Funahashi (1991), state that CD56+CD16+ NK cells are the most lytic subpopulation. Srour *et al.* (1990), state that CD3-(CD16+CD56+)CD57-CD8+ cells were most effective in mediating cytolysis against K562 cells. It is therefore possible that alterations in NK subsets, as a result of the disease or chemotherapy, may have impacted on the level of cytotoxicity obtained. Given these limitations the results need to be interpreted with caution. However,

the data would support reduced lytic activity of NK cells in LG-NHL patients at diagnosis and following chemotherapy as being either due to :-

1. Reduced lytic activity of CD56+ NK cells, as was found in CD56+ sorted cells in acute lymphoblastic leukaemia patients at diagnosis (Afify *et al.*, 1990).
2. Selective expansion of CD56+CD16- NK cells, the least lytic subset, which has been described in patients with bronchogenic carcinoma (LeFever and Funahashi, 1991). Dual fluorescent staining using both CD56 and CD16 antibodies would have been necessary to more definitively resolve the mechanism accounting for the findings reported in this study.

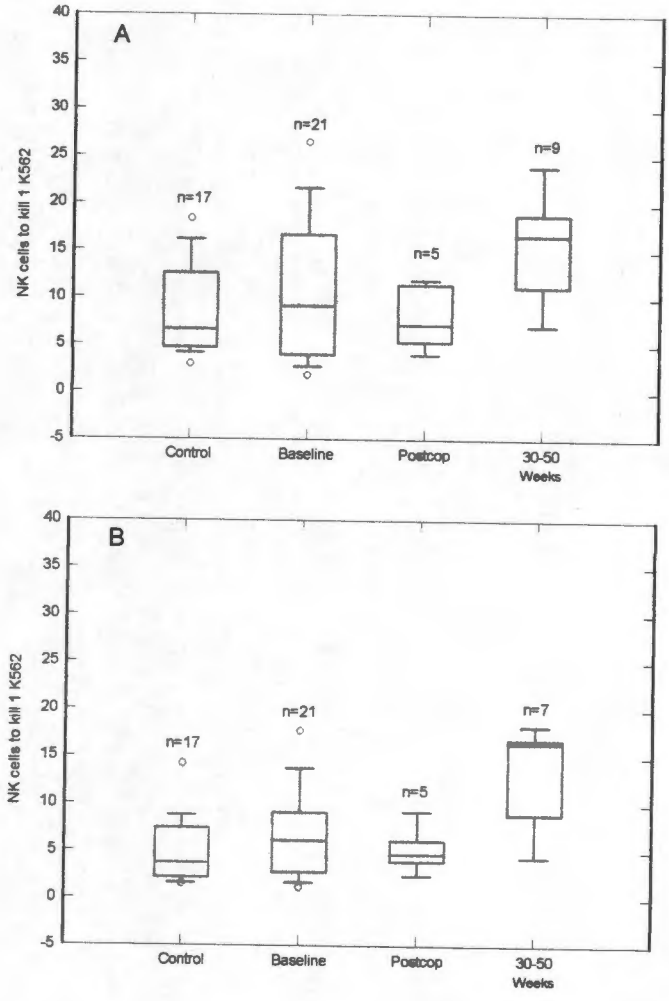


FIGURE 12. Effectivity of NK cells and effectivity of α-IFN enhanced NK cells in controls and patients over time.

NKA of PBMC from LG-NHL patients was evaluated in a standard 4 hour ^{51} chromium release assay against K562. By utilising the phenotypic analysis in determining the % NK cells present in the peripheral blood, and with the addition of a known number of lymphocytes in the NK assay, it was possible to determine the number of NK cells required to kill 1 K562 target cell (A). This figure is indicated on the Y-axis (A). In a similar manner the α-IFN enhanced NKA of PBMC from LG-NHL patients was evaluated with α-IFN (1 X 10^4 U/ml) incorporated into the NK assay. The effectivity of α-IFN enhanced NK cells was determined (B). This figure is indicated on the Y-axis (B). On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and 30-50 weeks after the commencement of treatment. Less patients are indicated in Figure 12 as compared to Figure 11, due to the fact that not all patients had both NKA and % NK cell number assessed due to insufficient cell recovery from peripheral blood samples.

3.2.3. MORPHOLOGICAL FEATURES OF NK CYTOTOXICITY

In an attempt to validate the standard chromium release cytotoxicity assay, the morphological features of NK cytotoxicity against K562 (Figure 13) and Daudi (Figure 14) at the termination of a four hour NKA assay (without the addition of ^{51}Cr) were examined microscopically. Trypan blue was added and cells counterstained with 0.5% carbol fuchsin. Although NK cells make contact and are interacting with the Daudi target cells, these target cells are not lysed (Figure 14). This can be compared to Figure 13, where the NK cells are both interacting with and lysing the K562 target cells.

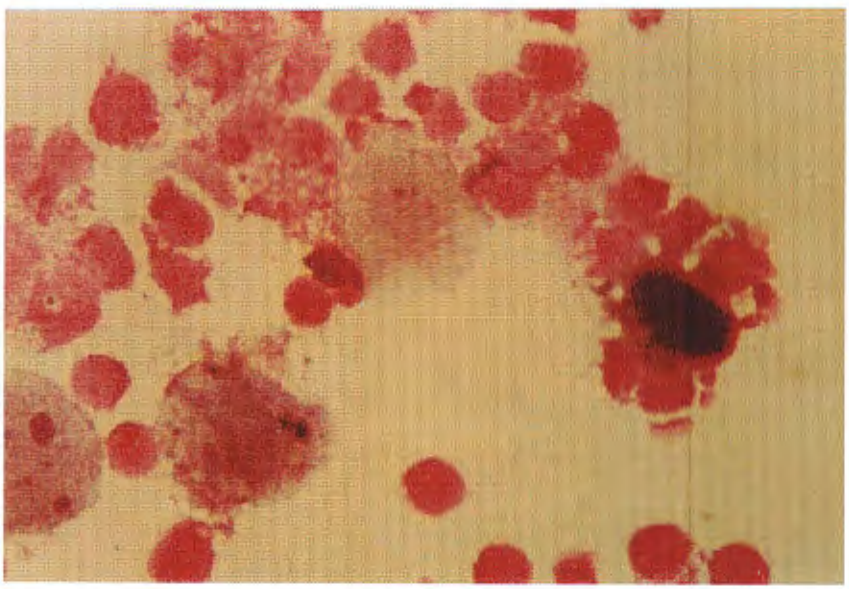


FIGURE 13. Morphological features of NK cytotoxicity assay against K562.
A 4 hour cytotoxicity assay was carried out as usual, but without the addition of sodium chromate. At the termination of the 4 hour period, 100 µl of supernatant was removed and 100 µl of trypan blue added. 100 µl of this sample was then subjected to cytospin slide centrifugation, air dried and counterstained with 0.05% carbol fuchsin. Viable cells are stained reddish-pink and non-viable cells blue-black, due to the uptake of trypan blue. A non-viable K562 cell can be seen in close contact with NK cells. Magnification X 1000.

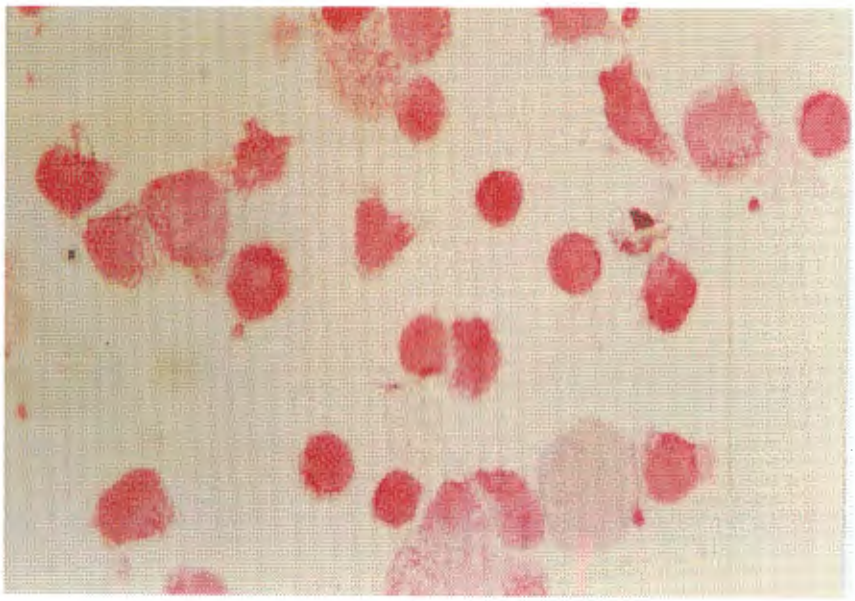


FIGURE 14. Morphological features of NK cytotoxicity against Daudi.
A 4 hour cytotoxicity assay was carried out as usual, but without the addition of sodium chromate. At the termination of the 4 hour period, 100 μl of supernatant was removed and 100 μl of trypan blue added. 100 μl of this sample was then subjected to cytospin slide centrifugation, air dried and counterstained with 0.05% carbol fuchsin. Viable cells are stained reddish-pink and non-viable cells would have stained blue-black, due to the uptake of trypan blue. Viable Daudi cells are in close contact with NK cells. Magnification X 1000.

3.2.4. ANALYSIS OF LAK AGAINST K562 AND DAUDI

LAK against K562 and Daudi is indicated in Figure 15 A and B respectively. Although only the percentage cytotoxicity at an effector to target ratio of 3 to 1 has been shown, it should be emphasised that all other effector to target ratios gave similar trends. LAK against K562 at baseline was found to produce comparable levels of cytotoxicity in both the patient (41.07 ± 20.93) and control groups (45.47 ± 17.73). These findings confirm those of Caldera and co-workers (1992), who reported that generation of LAK activity against K562 and Daudi was both possible and comparable in both responsive and unresponsive patients. No conclusions can be drawn from the LAK activity over time due to a wide spread of data and relatively few patient numbers, although the general trend appears to be an increase in the LAK against K562 up until 70 weeks after which there is a decline. No differences in any of the results were found to be statistically significant.

LAK against Daudi (Figure 15-B) was slightly depressed in patients at diagnosis (24.45 ± 17.15) compared to controls (32.74 ± 16.26). All patients tested were able to generate LAK activity against the NK resistant but LAK sensitive cell line Daudi. This confirmed the findings of Zamkoff and co-workers (1990), as discussed in the Introduction, where it was stated that LAK against Daudi was slightly depressed compared to controls, but not significantly so. Due to insufficient lymphocyte recovery in some patients, LAK against Daudi

was not performed, resulting in fewer patients in Figure 15-B than in Figure 15-A. As a result of this, only three patients are available at 30-50 weeks (27.28 ± 9.4) and the spread is quite large making any accurate observations impossible. It would be unwise therefore to speculate on the trend. Once again no differences in results are statistically significant.

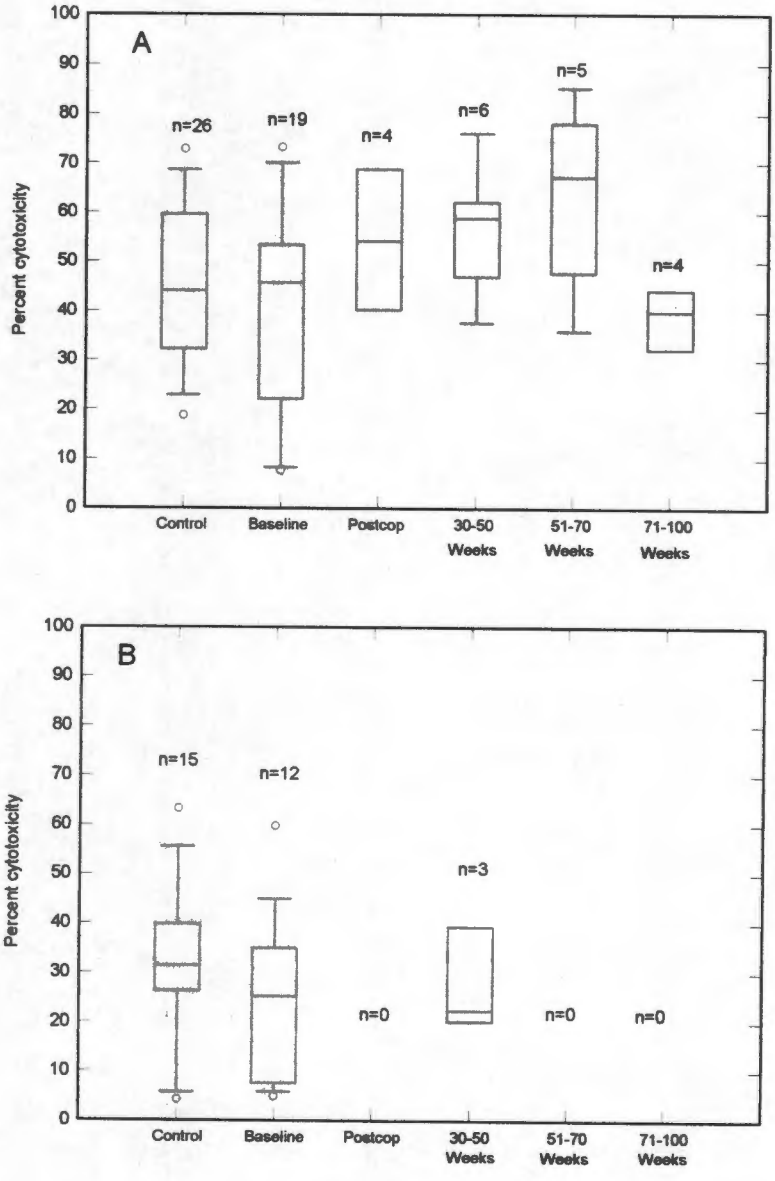


FIGURE 15. LAK against K562 and Daudi in controls and patients over time.
LAK cells were generated by culturing 6-10 X 10^6 PBMC at 10^6/ml in 10% human AB serum in RPMI, supplemented with 100 U/ml recombinant human IL-2 for 7 days. LAK activity was evaluated in a standard 4 hour ^{51} chromium release assay against K562 (A) and Daudi (B). On the Y-axis percentage cytotoxicity can be seen at an effector to target ratio of 3 to 1. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment. The LAK against Daudi is only indicated for baseline and 30-50 weeks after treatment due to insufficient lymphocyte recovery in many patients.

3.2.5. PHENOTYPIC EVALUATION OF BLOOD SAMPLES

The percentage of circulating CD3+ cells present in the peripheral blood of patients prior to the commencement of treatment (Figure 16) (57.25 ± 18.45) is statistically significantly lower than controls (73.69 ± 8.91) ($p<0.01$). Patients' percentage CD3+ cells are found to reduce at postcop and then increase until 51-70 weeks (78.93 ± 9.69), after which there is a reduction (65.67 ± 12.61). The percentage of CD3+ cells in patients at baseline are statistically significantly different from 30-50 weeks ($p<0.01$) and 51-70 weeks ($p=0.01$). The difference between postcop and 30-50 weeks is also significant ($p<0.05$), as well as the difference between postcop and 51-70 weeks ($p<0.05$). The difference between 30-50 weeks ($p<0.05$) and 71-100 weeks is also significant ($p<0.05$) as is the difference between 51-70 and 71-100 weeks ($p<0.05$). The difference between controls and postcop is also significant ($p<0.05$). Possible reasons for these important findings are discussed after the results for all T-cell subsets have been given.

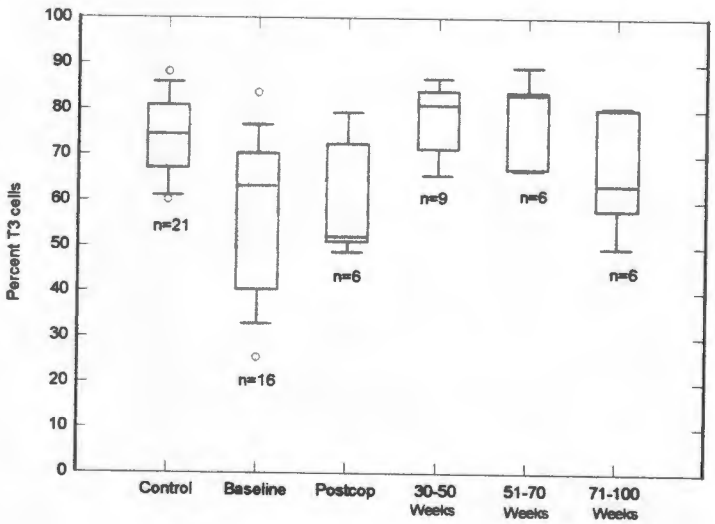


FIGURE 16. Percentage circulating CD3+ cells present in PBMC of controls and patients over time.

Flow cytometric analysis of PBMC was performed enabling the determination of % CD3+ cells present in the peripheral blood. On the Y-axis percentage CD3+ cells can be seen. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment.

The percentage of CD4+ cells present in the peripheral blood of patients at baseline (Figure 17) (36.28 ± 14.7) is lower than normal controls (43.36 ± 7.31), although not significantly so.

A very slight reduction in percentage CD4+ cells is found at postcop (34.05 ± 3.64) and then an increase at 30-50 weeks (41.33 ± 11.12) until 51-70 weeks (48.77 ± 11.88), after which at 71-100 weeks (31.8 ± 8.77), the percentage of CD4+ cells decreases. The percentage of CD4+ cells between controls and patients at postcop ($p<0.01$) and 71-100 weeks ($p<0.01$) are significantly different. The difference between patients' percentage CD4+ cells at postcop and 51-70 weeks is significant ($p<0.01$), as to is 51-70 and 71-100 weeks ($p<0.05$). Bergmann *et al.* (1985) report a dramatic reduction in CD4+ cells following either chemotherapy or radiotherapy. A similar reduction was not observed in this current study.

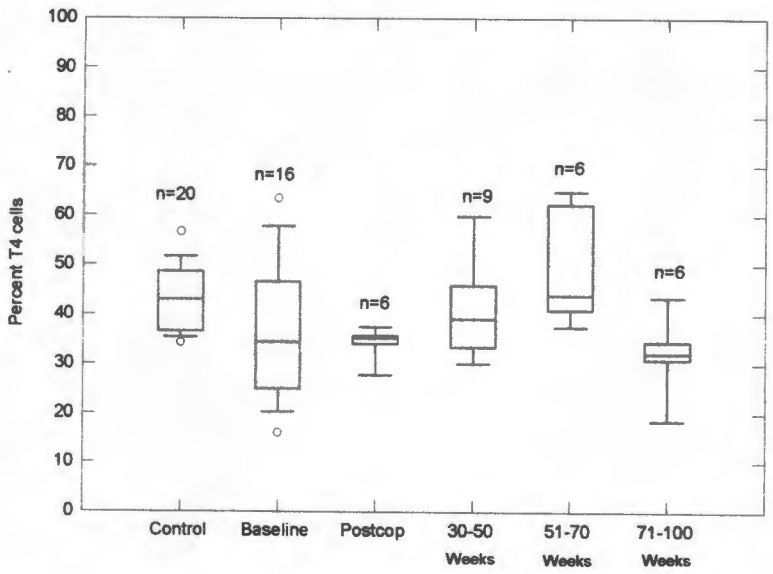


FIGURE 17. Percentage circulating CD4+ cells present in PBMC of controls and patients over time.

Flow cytometric analysis of PBMC was performed enabling the determination of % CD4+ cells present in the peripheral blood. On the Y-axis percentage CD4+ cells can be seen. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment.

Patients at diagnosis have significantly less percentage CD8+ cells present in the peripheral blood (Figure 18) (16.74 ± 8.98) as compared to controls (24.18 ± 5.75) ($p<0.05$). The circulating percentage CD8+ cells are found to increase at postcop (22.73 ± 14.14) until 30-50 weeks (30.58 ± 9.88) after which a reduction at 51-70 weeks (28.13 ± 8.66) and slight increase at 71-100 weeks is then found (28.93 ± 9.81). The difference between the baseline results and 30-50 weeks, 51-70 weeks and 71-100 weeks is significant with p values of 0.005; 0.04 and 0.02 respectively.

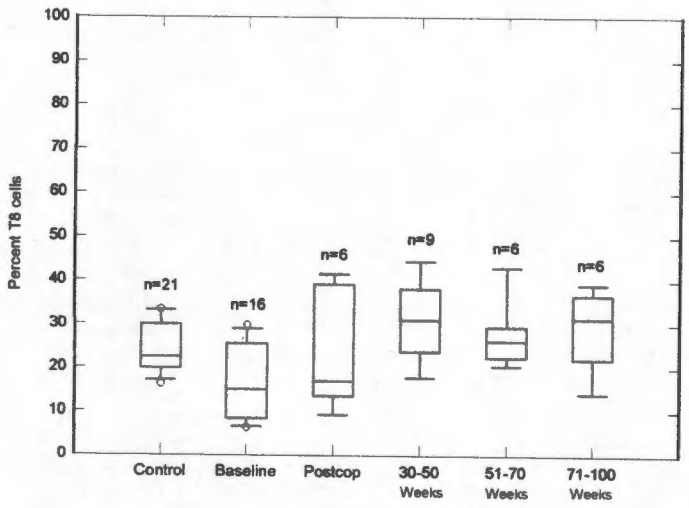


FIGURE 18. Percentage circulating CD8+ cells present in PBMC of controls and patients over time.

Flow cytometric analysis of PBMC was performed enabling the determination of % CD8+ cells present in the peripheral blood. On the Y-axis percentage CD8+ cells can be seen. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment.

As discussed in the Introduction, Burger and colleagues (1990), concluded that in both high and low grade NHL, patients were found to have a decrease in CD3+, CD4+, Leu-7 (an NK and T-cell subset marker) and NK cells, with no difference in CD8+ cells. These findings were dependent on the pathological state of the patient. The results in this study appear to partially confirm those of Burger and co-workers, however CD8+ cells were also markedly reduced.

It must be emphasised that the changes in lymphocyte subsets are relative changes only, as the percentages of these cells and not absolute numbers were determined. It must be bone in mind that as the percentage of one cell subset increases, this would inevitably cause a reduction in another subset. Given these limitations, the possible reasons for reduced T cells and their subsets at diagnosis could be that:-

1. The cells are not present in the peripheral blood as they have translocated to the site of pathology.
2. The occurrence of bulk disease in the bone marrow i.e. the malignant clone resulting in marrow replacement. This would result in less precursor T cells and consequently fewer percentages of CD3+, CD4+ and CD8+ cells.
3. As mentioned in the Introduction, depending on the type and stage of evolution of NHL, malignant cells may be present in the peripheral blood, similar to the lymphocyte leukaemic picture (Babior and Stossel, 1994). If this is the case, the reduction in percentages of CD3+ cells present in the peripheral blood may be as a result of the malignant spill-over into the peripheral blood resulting in a reduction of percentage CD3+ cells. The leukaemic picture is often present in NHL patients classified as Category A of the Working Formulation, which accounts for only approximately 10% of patients in this study. As the majority of patients are Category B and C, this explanation is not as valid as previous ones discussed.

The general trend for the percentage of circulating CD3+, CD4+ and CD8+ cells is found to increase until approximately 51-70 weeks after which time there is a substantial decrease in the percentage of CD3+ and CD4+ cells. The percentage of CD8+ cells is found to plateau off after 51-100 weeks. The reason for the reduction in the percentage CD3+ cells at postcop is undoubtedly due to chemotherapy. The increase in percentage CD3+ after postcop and percentage CD4+ and CD8+ cells after baseline, may be due to a reduction in the malignant cells in the bone marrow allowing for more precursor lymphocytes. More precursor cells in the bone marrow would result in more T cells being present in the peripheral blood. This finding would be largely dependent on the amount of myelosuppression caused by the chemotherapy. It is important to remember that changes in the peripheral blood cell counts lag behind changes in the bone marrow production pools as previously mentioned (Bodensteiner and Doolittle, 1993; Dorr and Fritz, 1982). Another possibility is that as the chemotherapy begins to control the lymphoma cells at the site of pathology, there is no longer a need for these cells to leave the peripheral blood and translocate to the site of pathology. This would result in the presence of more T cells in the peripheral blood which was seen after postcop. A further explanation could be that as the chemotherapy begins to control the number of malignant cells present in the peripheral blood, this would allow the T cell numbers to stabilise.

It must be emphasised that the reduction in the percentage of circulating CD3+ and CD8+ cells at diagnosis is not due to the fact that these patients are lymphopenic, as only five of thirty patients tested demonstrated reduced absolute lymphocytes (Table 12, 13 and 14), which would not account for the statistically significant differences that were obtained. Of the two lymphopenic patents on whom T-cell subsets were analysed, one patient had a reduction in the percentages of CD3+, CD4+ and CD8+ cells and the other a reduction in percentage CD8+ cells. One patient who was both lymphopenic and leukopenic had normal percentages of CD3+, CD4+ and CD8+ cells. Three of the patients presenting with normal numbers of both lymphocytes and leukocytes had a reduction in the percentages of CD4+ cells, another three a reduction in the percentages of CD8+ cells and one patient a reduction in percentages of CD3+, CD4+ and CD8+ cells. One patient with normal absolute leukocytes and lymphocytes had increased percentages of CD3+ cells. The most interesting finding, was that four patients with increased lymphocytes and leukocytes had reduced percentages of CD3+, CD4+ and CD8+ cells. It is unfortunate that a full blood count and a differential count were not performed simultaneously with phenotypic evaluation, which would have enabled a more conclusive assessment of absolute cell numbers as well as percentages of cells, especially in the light of these above-mentioned findings. Although full blood count, differential count and phenotypic evaluation was undertaken in most of the patients, these were performed at different times, making an accurate evaluation of absolute numbers of T-cell subsets impossible.

The ratio between the percentage of circulating CD4+ and CD8+ (CD4:CD8) (Figure 19) between patients' baseline results (2.82 ± 1.95) and controls (1.94 ± 0.64) is found to be higher, although not significantly so. The ratio has diminished by postcop (2.09 ± 1.34) and more so by 30-50 weeks (1.57 ± 0.92). There is a slight increase in the mean at 51-70 weeks (1.89 ± 0.81) and a reduction in this value at 71-100 weeks (1.48 ± 0.61). The difference between the CD4:CD8 ratio from baseline and 30-50 ($p<0.05$) and 71-100 weeks ($p<0.05$) is significant. The difference between the control results and patient results at 71-100 weeks is also significant, with $p<0.05$.

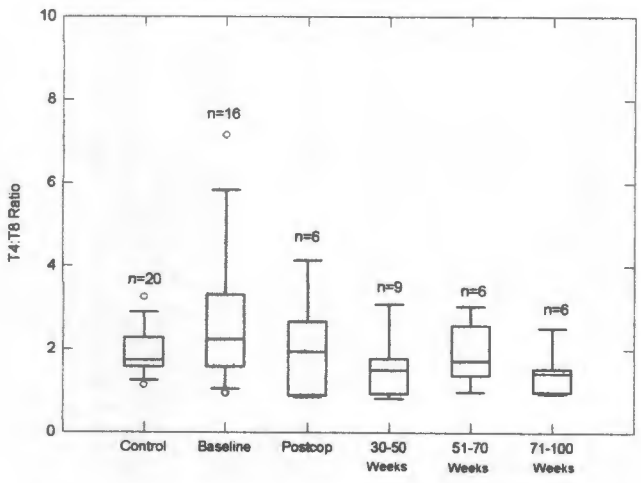


FIGURE 19. The ratio of % CD4+ cells to % CD8+ cells present in PBMC of controls and patients over time.

Flow cytometric analysis of PBMC was performed enabling the determination of % CD4+ cells present and % CD8+ cells present in the peripheral blood. The ratio of % CD4+ cells to % CD8+ cells was determined. On the Y-axis the ratio of CD4:CD8 cells can be seen. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment.

There are significantly more percentage NK cells present at baseline in the patients (Figure 20) (20.65 ± 12.08) when compared to controls (11.59 ± 5.77) ($p=0.01$), as determined by staining CD56+ which is a known NK cell marker. A reduction is found in this value at 30-50 weeks (11.79 ± 6.75) by which time the result is very similar to that of normal controls. There is a steady increase in the percentage of NK cells until 71-100 weeks (21.31 ± 7.64). The difference between patients' baseline results and 30-50 weeks is significant, with $p<0.05$. It is intriguing to note that a similar statistically significant reduction in percentage NK cells and NKA is seen at 30-50 weeks. This finding is possibly as a result of chemotherapy causing a reduction in the percentage of circulating NK cells, which would further result in reduced NKA. The difference between 30-50 weeks and 71-100 weeks (21.31 ± 7.64) is also significant ($p<0.05$). The difference between controls (11.59 ± 5.77) and postcop patients (21.24 ± 11.27) as well as patients at 71-100 weeks (21.31 ± 7.64) after the commencement of treatment is significant, with $p<0.05$ and $p<0.01$ respectively.

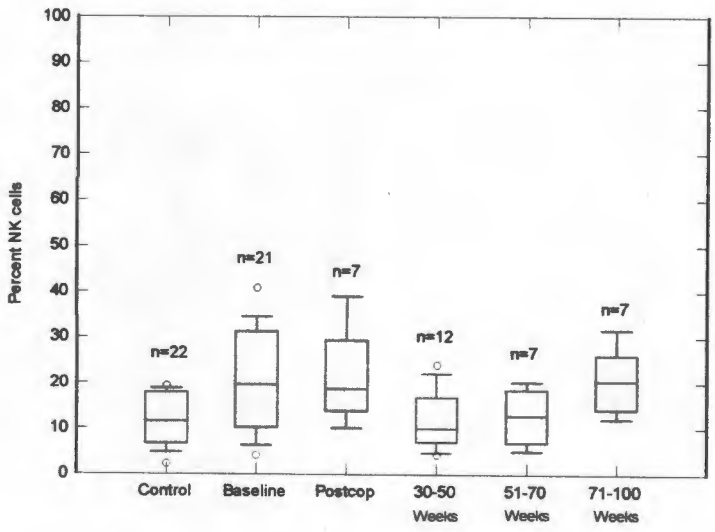


FIGURE 20. Percentage circulating NK cells present in PBMC of controls and patients over time.

Dual parameter flow cytometric analysis of PBMC was performed enabling the determination of % CD56+ (NK) and % CD3- cells present in the peripheral blood. On the Y-axis percentage NK cells can be seen. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment.

NK cells have been proposed as the first line of defence against tumour cells (Caldera *et al.*, 1992). Data indicates that NK cell anti-tumour activity is highly expressed in the blood stream thus preventing metastasis via this route. This highlights the importance of NK cells in the control of metastasis and the growth of primary tumours (Reynolds and Wiltrout, 1989). It has been proposed that NK cells recognise and kill tumour cells during the blood-borne phase of metastasis, but when there is a large tumour burden, NK cells are not as competent. This is possibly due to the fact that their lytic capacity is overwhelmed, or depressed due to tumour release of prostaglandins (Shepard and Shek, 1995; Reynolds and Wiltrout, 1989). The increase in the percentage of NK cells in the peripheral blood at diagnosis of patients in this study when compared to normal controls, is possibly an attempt by NK cells to overcome the tumour burden.

Most of the evidence on NK cell function in antitumour immunity has been gained from experimental animals. In mice, it was found that NK cells were able to inhibit NK sensitive tumour cells *in vivo*. *In vivo* depletion of NK cells in experimental animals has resulted in depressed immunity against tumours (Kos and Engleman, 1996). NK cells have been found to accumulate at the sites of inflammation in both primary and transplanted tumours

(Reynolds and Wiltrout, 1989). 'Beige' mice with an NK defect, appear to be prone to the development of lymphoma, suggesting an important role of immune surveillance for NK cells (Krzanowski, 1991). In this study, the increase in percentage of NK cells in the peripheral blood could be an attempt by the host to overcome the malignant cells. It is dissappointing that staining was only undertaken on a single NK cell marker (CD56). Had dual staining been carried out on both CD56 and CD16 markers, more information could have been gained on the lytic ability of these cells. As previously mentioned, the CD56+CD16+ NK cells are more lytic than CD56+CD16- NK cells (LeFever and Funahashi, 1991). The finding of a particular cell phenotype however does not imply fully functional activity as indicated by Urba and co-workers (1987). Kurago and co-workers (1995) report that NK cells are only able to control small numbers of injected tumour cells, whereas CTL are able to eliminate large numbers of immunogenic tumour cells. These cells therefore appear to perform complementary functions to each other. Although in this study the percentage of NK cells was increased in the bloodstream, the percentage of CD8+ cells was depressed. Possibly this complementary function was not working as effectively as it should have been (Moretta *et al.*, 1996).

The decrease in percentage CD3+ cells and the increase in percentage NK cells of patients at diagnosis when compared to normal controls could also be attributed to secondary reciprocal changes in the cell types sharing the common peripheral blood compartment.

A comparison was made between percent NK cells and cytotoxicity in both controls and patients and no correlation was found (data not shown). Whiteside (1991), concluded that in normal healthy individuals, NKA did not appear to correlate with the number of circulating NK cells. This finding is confirmed in this current study.

3.2.6. MITOGEN AND ANTIGEN PROLIFERATION

The mean of the PHA stimulation index (SI) [a ratio of PHA counts per minute (cpm's) to background cpm's] for patients at diagnosis (349.74 ± 233.68) and prior to the commencement of treatment is similar to that of normal controls (340.64 ± 362.67) (Figure 21). This is a highly relevant finding especially in the light of the fact that percentage CD3+ cells are decreased at diagnosis in the patient group. There is a dramatic reduction in PHA SI until 51-70 weeks (62.03 ± 40.63), after which there is a slight increase in this value at 71-100 weeks (198.8 ± 161.39). The baseline results and 51-70 week results are significantly different ($p<0.05$), as too are the control and 51-70 week results ($p<0.01$). As the percentage CD3+ cells increase after postcop, it is possible that this reduction in PHA SI could reflect a T-cell defect, induced by chemotherapy.

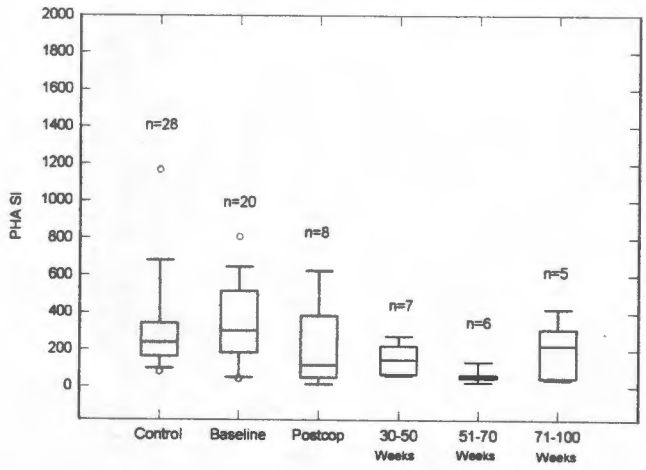


FIGURE 21. PHA stimulation of PBMC in controls and patients over time.
PBMC were resuspended at 1 X 10^6/ml in RPMI supplemented with 10% AB human serum. Mitogenic stimulation was assessed in response to 5.75 X 10^{-3} mitogenic units/ml of PHA after 3 days incubation. Thereafter tritiated thymidine incorporation was measured in a beta counter. On the Y-axis the ratio of PHA cpm's to background cpm's (PHA SI) are given. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment.

The PPD SI of controls (66.4 ± 73.3) (Figure 22) is significantly higher than the patients' baseline results (64.2 ± 166.2) ($p<0.01$). Patients' results drop dramatically at postcop (2.6 ± 3.34) and remain low until 51-70 weeks (4.7 ± 5.4) after which there is an increase in the mean PPD SI at 71-100 weeks (22 ± 47.15). The difference between controls and patients at the following time points are significant :- postcop ($p<0.001$); 30-50 weeks ($p<0.01$); 51-70 weeks ($p<0.01$) and 71-100 weeks ($p<0.05$). It is interesting to note that this PPD antigen-specific proliferation is profoundly depressed in patients at diagnosis and remains inhibited at all time points. This could possibly indicate a defect in either the responding cells or the antigen presenting cells, or both.

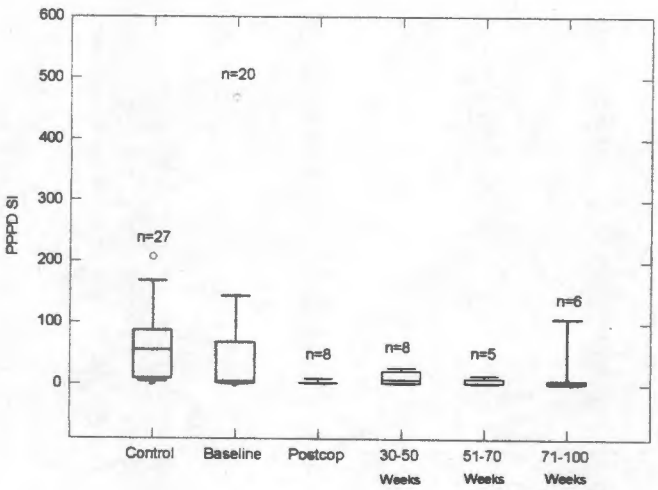


FIGURE 22. PPD stimulation of PBMC in controls and patients over time.
PBMC were resuspended at 1 X 10^6/ml in RPMI supplemented with 10% AB human serum. Antigenic stimulation was assessed in response to 3 μg/ml of PPD after 6 days incubation. Thereafter tritiated thymidine incorporation was measured in a beta counter. On the Y-axis the ratio of PPD cpm's to background cpm's (PPD SI) are given. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 71-100 weeks after the commencement of treatment.

The SK-SD SI for controls (71.75 ± 104.2) is slightly higher than that of patients at baseline (40.3 ± 52.64) though not significantly so (Figure 23). There are no significant differences obtained between controls and patients at any of the different time points. The patient number for the measurement of SK-SD proliferation is greatly reduced at diagnosis, as compared to PHA or PPD proliferation, due to the fact that there was insufficient cell recovery in some patients. This fact alone may account for the finding that SK-SD is very similar in both the control and patient group.

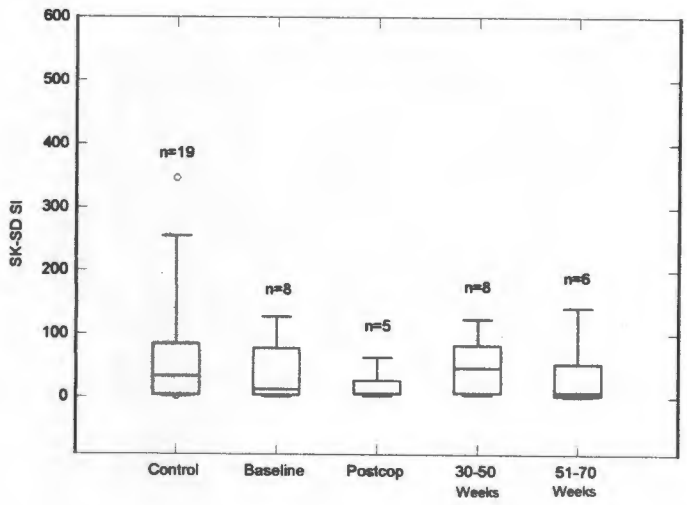


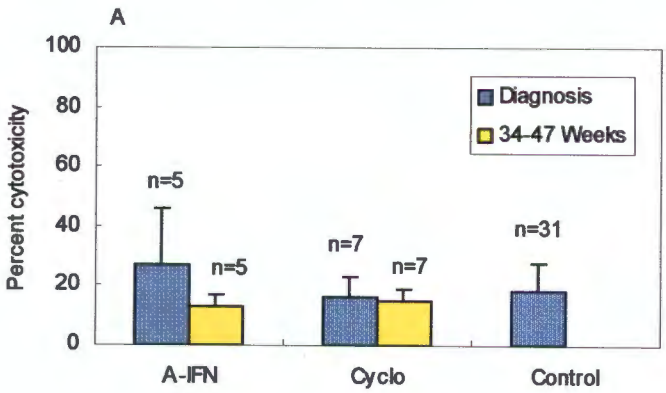
FIGURE 23. SK-SD stimulation of PBMC in controls and patients over time.
PBMC were resuspended at 1 X 10^6/ml in RPMI supplemented with 10% AB human serum. Antigenic stimulation was assessed in response to heat treated 250 U/ml streptokinase; 62.5 U/ml streptodornase after 6 days incubation. Thereafter tritiated thymidine incorporation was measured in a beta counter. On the Y-axis the ratio of SK-SD cpm's to background cpm's (SK-SD SI) are given. On the X-axis, the normal controls are indicated first, with patients' baseline results next (at diagnosis and prior to the commencement of treatment). Thereafter the patients' results are given for postcop and at various time intervals until 51-70 weeks after the commencement of treatment.

3.2.7. ANALYIS OF THE EFFECT OF TREATMENT ON NKA AND α-IFN ENHANCED NKA

NKA of patients at baseline and prior to the commencement of α-IFN treatment (26.9 ± 19.14) are compared with the same patients 34-47 weeks after the commencement of treatment (12.98 ± 6.28) (Figure 24-A). These patients are compared to baseline results of untreated patients who are subsequently treated with cyclophosphamide (16.39 ± 8.67) and the same patients 34-47 weeks into treatment (14.89 ± 14.29). It must be borne in mind that α-IFN and cyclophosphamide treatment commenced at approximately 6 weeks. NKA of normal controls (18.43 ± 10.25) are also indicated. The NKA of patients at 34-47 weeks treated with α-IFN is found to be depressed compared to baseline, although this difference is not statistically significant. These results confirm that of other researchers who report that the continued and frequent use of *in vivo* α-IFN treatment often causes a decline in NKA (Reynolds and Wiltrout, 1989; Ozer *et al.*, 1983; Maluish *et al.*, 1983). Patients treated with cyclophosphamide, on the other hand show very similar NKA at baseline and 34-47 weeks.

The same patient groups as well as time points are compared as in Figure 24-A, but with α-IFN enhanced NKA. Patients that have been treated with α-IFN have been found to have

severely depressed NKA boosted by α-IFN (12.55 ± 7.28) when compared to their own baseline results (39.97 ± 24.97) (Figure 24-B). Using the Wilcoxon signed-ranks test for paired data, this difference was found to be significant with $p<0.05$. This is an extremely interesting finding and may indicate that the *in vivo* administration of α-IFN has rendered the NK cell insensitive to further stimulation by α-IFN *in vitro*. NK cells appear to be maximally boosted *in vivo*. This may imply that down-regulation of α-IFN receptors has occurred resulting in a decrease of α-IFN augmented NKA. Cyclophosphamide patients were found to have very similar results at both baseline (23.5 ± 10.05) and 34-47 weeks after the commencement of treatment (23.42 ± 19.08). Control results (26.89 ± 10.08) have also been included which are slightly higher than those of cyclophosphamide treated patients.



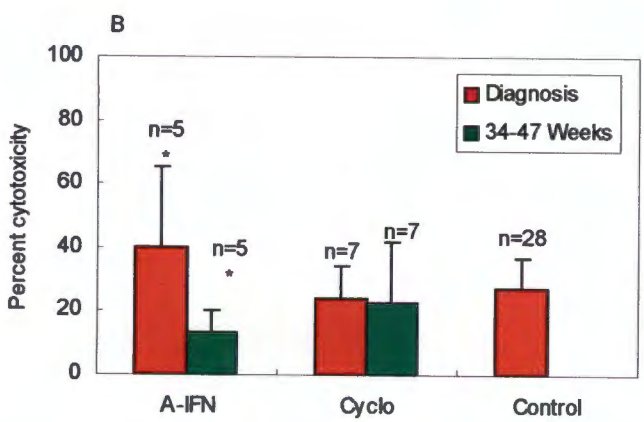


FIGURE 24-A. A comparison of NKA in controls and patients at diagnosis and 34-47 weeks after treatment.

FIGURE 24-B. A comparison of α-IFN enhanced NKA in controls and patients at diagnosis and 34-47 weeks after treatment.

NKA (A) or α-IFN enhanced NKA (B) of PBMC from LG-NHL patients was evaluated in a standard 4 hour ^{51} chromium release assay against K562 at diagnosis and prior to the commencement of treatment. α-IFN (1 X 10^4 U/ml) was incorporated into the NK assay for α-IFN enhanced NKA (Figure 24-B). These same patients were then analysed 34-47 weeks after the commencement of treatment. On the Y-axis percentage cytotoxicity can be seen at an effector to target ratio of 10 to 1. On the X-axis, α-IFN (A-IFN) patients at diagnosis is shown, followed by the same patients 34-47 weeks after the commencement of α-IFN treatment. Similarly cyclophosphamide patients at diagnosis is shown, followed by the same patients 34-47 weeks after the commencement of cyclophosphamide treatment. The controls NKA (Figure 24-A) or α-IFN enhanced NKA (Figure 24-B) is indicated last on the X-axis. Blue bars are baseline results and yellow bars indicate the NKA after 34-47 weeks of treatment (Figure 24-A). Red bars are baseline results and green bars indicate the NKA after 34-47 weeks of treatment (Figure 24-B). Standard deviation is indicated as error bars. Stars indicate statistically significant differences ($p<0.05$).

3.2.8. PHENOTYPIC ANALYSIS AND ITS COMPARISON TO TREATMENT RESPONSES

A bar graph of percentage CD3+, CD4+, CD8+ and NK cells present in the peripheral blood of all patients compared to normal controls is shown in Figure 25. The difference between the percentage of circulating CD3+ in patients at diagnosis (57.25 ± 18.45) and controls (73.69 ± 8.91) is found to be statistically significant ($p<0.01$), as also the percentage CD8+ in patients at diagnosis (16.74 ± 8.99) and controls (24.18 ± 5.75 ($p<0.05$). Similarly the percentage circulating NK cells in patients at diagnosis (20.65 ± 12.08) and percentage NK cells in normal controls (11.59 ± 5.77) is also significant ($p<0.05$). The percentage of CD4+ cells is also decreased in patients (36.28 ± 14.7) as compared to normal controls (43.36 ± 7.31), although not significantly so.

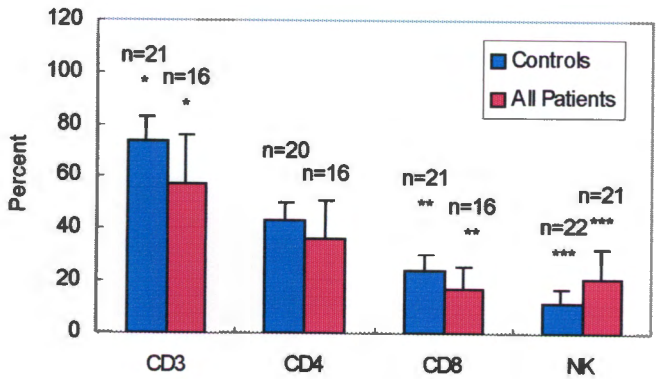


FIGURE 25. A comparison of % CD3+, CD4+, CD8+ and NK cells present in the peripheral blood of controls compared to all patients.
Flow cytometric analysis of PBMC was performed enabling the determination of % CD3+, CD4+, CD8+ and NK cells present in the peripheral blood at diagnosis and prior to the commencement of treatment. On the Y-axis the percentage of cells positive for the different phenotype is indicated. On the X-axis, CD3, CD4, CD8 and NK cells are indicated, with controls first, followed by patients. Stars indicate a statistically significant difference. The difference between % CD3+ in controls and patients is significant ($p<0.01$), as is the % CD8+ in patients at diagnosis ($p<0.05$). Similarly the percentage circulating NK cells in patients at diagnosis is also significant ($p<0.05$). Turquoise bars represent controls and magenta bars patients. Standard deviation is indicated as error bars.

Figure 26 A-D is a bar graph of the above-mentioned parameters, but in this case the patients have been divided into the different treatment schedules of cyclophosphamide, α-IFN and miscellaneous patients (those not treated according to protocol). Patients are then further classified as responding patients (this includes partial and complete response) and non-responding patients (this includes minimal response, progressive disease, transformation to a higher grade of NHL and relapsing patients) (Czader *et al.*, 1996). The mean percentage circulating CD3+ cells in controls (74 ± 9) is significantly more than either cyclophosphamide non-responding patients (35 ± 0) ($p<0.05$) or miscellaneous non-responding patients (46 ± 21) ($p=0.01$) (Figure 26-A). Cyclophosphamide responding

patients (72 ± 19), α-IFN non-responding patients (69 ± 7) and miscellaneous responding patients (66 ± 4) gave comparable results. The mean percentage circulating CD4+ cells in controls (43 ± 7) is significantly more than in cyclophosphamide non-responding patients (21 ± 1) ($p<0.05$) and significantly less than miscellaneous responding patients (55 ± 4) ($p=0.05$) (Figure 26-B). Cyclophosphamide non-responding patients have an increase in percentage CD4+ cells (53 ± 13) and α-IFN non-responding patients (34 ± 8) and miscellaneous non-responding patients (27 ± 10) less when compared to normal controls, although none of these differences reached statistical significance. Cyclophosphamide non-responding patients (12 ± 1) ($p<0.05$) and miscellaneous responding patients (9 ± 1) ($p<0.05$) had significantly fewer percentage CD8+ cells in the peripheral blood than normal controls (24 ± 6) (Figure 26-C). Cyclophosphamide responding patients (19 ± 10) and miscellaneous non-responding patients (15 ± 11) had less percentage CD8+ cells and α-IFN non-responding patients (26 ± 5) more than normal controls. Both cyclophosphamide (30 ± 15) and α-IFN (30 ± 6) responding patients had significantly more percentage circulating NK cells than controls (12 ± 6), with $p<0.05$ and $p<0.01$ respectively (Figure 26-D). Cyclophosphamide non-responding patients (18 ± 4), α-IFN non-responding patients (17 ± 11), miscellaneous responding patients (18 ± 2) and miscellaneous non-responding patients (12 ± 9) presented with comparable percentages of circulating NK cells present at diagnosis. Due to the fact that patient numbers in these subgroups are small, it would be unwise to speculate on the relevance of any of these differences.

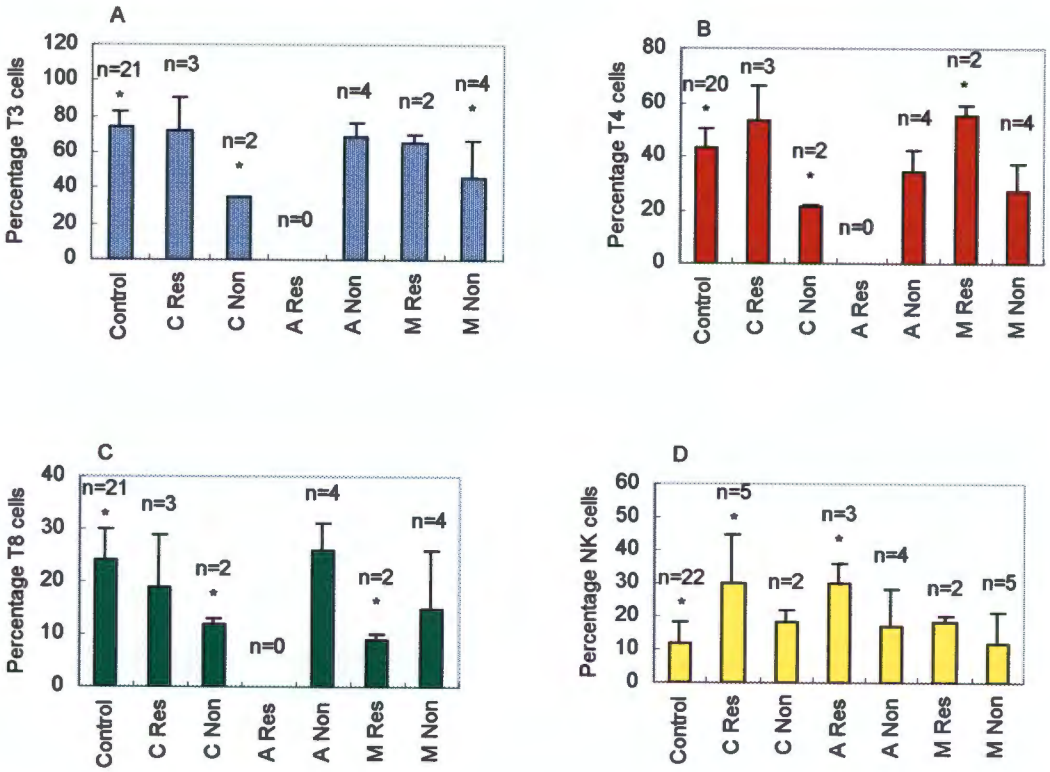


FIGURE 26. A comparison of % T-cell subsets and % NK cells present in the periphera blood at diagnosis in the different patient treatment schedules and their responses.

Flow cytometric analysis of PBMC was performed enabling the determination of % CD3+ (A), % CD4+ (B), % CD8+ (C) and % NK cells (D) present in the peripheral blood at diagnosis and prior to the commencement of treatment. On the Y-axes the percentage of cells positive for the different phenotype is indicated. On the X-axes the controls are indicated first, followed by cyclophosphamide responding patients (C Res), cyclophosphamide non-responding patients (C Non), α-IFN responding patients (A Res), α-IFN non-responding patients (A Non), miscellaneous responding patients (M Res) and miscellaneous non-responding patients (M Non). Responding patients include both a partial and complete response. Non-responding patients include minimal response, progressive disease, transformation to a higher grade of NHL and relapsing patients. α-IFN treated responding patients were only ascertained for percentage NK cells present at diagnosis due to insufficient cell recovery. Statistically significant differences have been indicated by stars. Standard deviation is shown as error bars.

3.2.9. PHENOTYPIC TRENDS OVER TIME IN RESPONDING AND NON-RESPONDING PATIENTS

A comparison was made between responding and non-responding patients over time in all treatment schedules for the following:- percentages CD3+, CD4+, CD8+ and NK cells present in the peripheral blood, the ratio of CD3+ to CD4+ cells and NKA. This was undertaken to ascertain whether a different trend could be detected between the different responses. Hernberg and co-workers (1997), found an increase in the CD4+ to CD8+ cell

ratio to be a good prognostic indicator and a reduction in this value a poor prognostic indicator in α-IFN treated renal cell carcinoma patients. In this current study, similar trends were detected for all the above-mentioned parameters (data not shown), except for percentage NK cells present in the peripheral blood. Responding patients showed a dramatic reduction in percentage NK cells over time, whereas non-responding patients had a gradual increase in this percentage (Figure 27). Although $r^2 = 0.59$ for responding patients and $r^2 = 0.21$ for non-responding patients, this trend may be an important indicator of response over time, if confirmed in a larger trial. This phenomenon could be explained by the fact that in responding patients, the NK cells have translocated to the site of pathology, whereas in non-responding patients this has not taken place. Literature appears to be contradictory in this regard, as Trinchieri (1992), states that following *in vivo* activation, NK cells migrate to affected areas and draining lymph nodes. Reynolds and Wiltrout (1989), state that NK cell number in the lymph nodes is low. Warren (1996) on the other hand report that NK cells do not circulate between the peripheral blood and lymph nodes. Pross and Lotzová (1993), state that NK cells are seldom present as tumour-infiltrating lymphocytes.

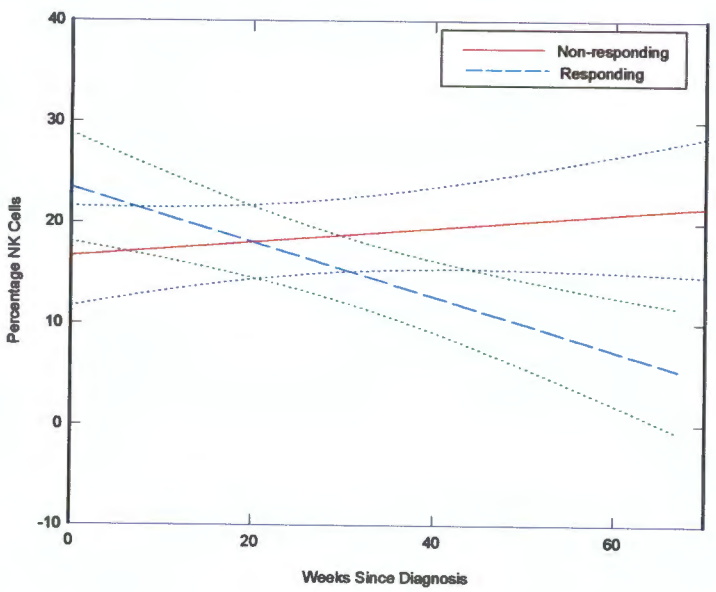


FIGURE 27. A comparison between % NK cells over time in responding and non-responding patients.

Flow cytometric analysis of PBMC was performed enabling the determination of % NK cells at diagnosis and prior to the commencement of treatment and this parameter was measured serially over time. Patients in all the different treatment schedules were included and responding patients were compared to non-responding patients. 95% confidence intervals are indicated. In the equation $Y = mx + c$, for responding patients, $m = -0.26965$ and $c = 23.435$ and $n = 16$. For non-responding patients, $m = 0.070425$ and $c = 16.602$ and $n = 11$.

3.2.10. CYTOKINE ANALYSIS

Only cyclophosphamide treated patients will be analysed for IL-2 levels, due to the fact that insufficient α-IFN treated patients were assessed for this parameter. Figure 28 is an indication of the amount of IL-2 present in plasma and Figure 29, the amount of IL-2 present in the supernatant fluid of PBMC after stimulation with the mitogen PHA (5.75×10^{-3} mitogenic units/ml). Both of these parameters were measured at diagnosis and prior to the commencement of any treatment. Random selection of patients from each treatment group was undertaken for IL-2 analysis. Very little IL-2 was found in the supernatant fluids and even less in the plasma. With such low values it is difficult to interpret any differences at all. In the PHA supernatants, the mean for IL-2 levels in normal controls (4.65 ± 2.6) was similar to that of cyclophosphamide responding patients (6.88 ± 5.58). The cyclophosphamide non-responding patients are found to have an increase in the mean of IL-2 levels (25.77 ± 43.34), but the range is too great to enable any relevant conclusions to be made. Whiteside (1994), discussed whether plasma or serum was more valuable for determining the amount of cytokine present. This author concluded that leukocytes including immune cells were activated during the clotting process and may release cytokines. As an alternative to plasma, it was recommended that cytokine analysis of supernatant fluid be performed after PHA stimulation of PBMC cells. In this study supernatant fluids were analysed in this way. A possible flaw in the method used in this study, was that the cells were not irradiated prior to PHA addition which would have prevented utilisation of IL-2. Alternatively, the IL-2 receptor could have been blocked with anti-IL-2 receptor antibody prior to the addition of PHA. If either of these two steps had been undertaken, more free IL-2 would possibly have been detected in the supernatant fluids.

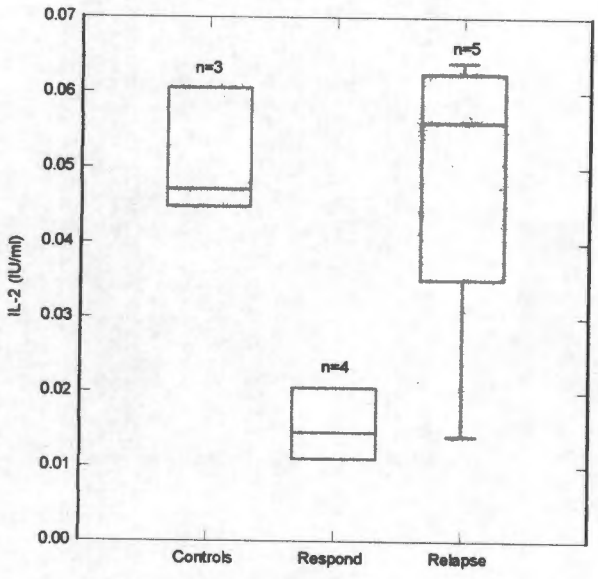


FIGURE 28. Concentration of IL-2 present in the plasma of controls and cyclophosphamide treated patients.

IL-2 concentrations were measured in the plasma of cyclophosphamide treated patients at diagnosis and prior to the commencement of treatment using a commercial immunoradiometric assay. IL-2 concentration is indicated as IU/ml on the Y-axis. On the X-axis, controls are indicated first, then cyclophosphamide responding patients (respond) and lastly cyclophosphamide non-responding patients (relapse).

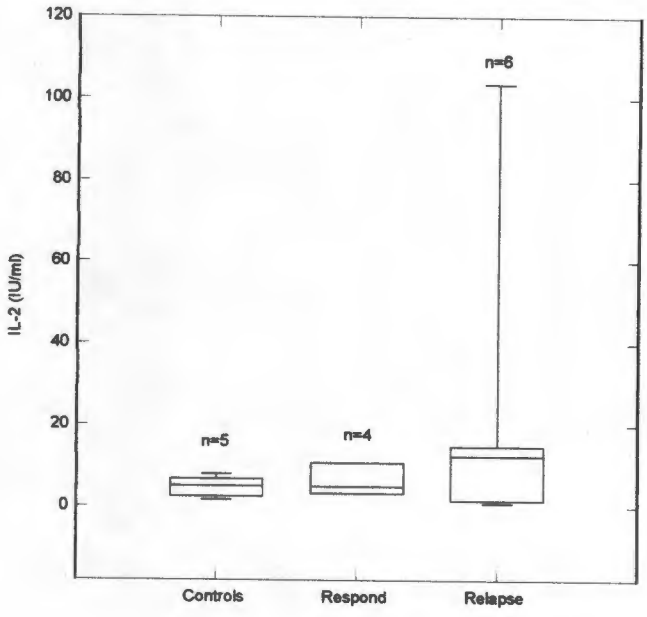


FIGURE 29. Concentration of IL-2 present in the supernatant of PHA treated PBMC in controls and cyclophosphamide treated patients.

PBMC (3 X 10^6/ml/well) at diagnosis and prior to the commencement of treatment were cultured in 10% human AB serum in RPMI and stimulated by 5.75 X 10^{-3} mitogenic units/ml of PHA for 24 hours. Supernatant fluids were then removed and IL-2 concentrations were then measured using a commercial immunoradiometric assay. IL-2 levels are indicated as IU/ml on the Y-axis. On the X-axis, controls are indicated first, then cyclophosphamide responding patients (respond) and lastly cyclophosphamide non-responding patients (relapse).

3.3.0. ANALYSIS OF PATIENTS ACCORDING TO TREATMENT SCHEDULES

3.3.1. ANALYSIS OF CYCLOPHOSPHAMIDE TREATED PATIENTS

Table 12 summarises the different parameters of category according to the Working Formulation, stage, age and sex of cyclophosphamide treated patients and their subsequent response. It was possible to adequately assess cyclophosphamide response in twelve patients. Fifty eight percent (7 patients) had a complete response and 42% (5 patients) a partial response following treatment (2 of which suffered a relapse). Seventeen percent of patients (2) who had previously demonstrated a partial response suffered a relapse. All of the cyclophosphamide treated patients were diagnosed as being at stage IV. Greater than 60-70 years is also used as a poor prognostic indicator in other trials, but 57% (4) of patients with a complete response and 14% (1) with a partial response were between 60-70 years of age. One of the two patients who relapsed was 40 years of age and the other 63 years of age . Male gender is also used as an unfavourable prognosis. In the seven complete

responding patients, three were male (43%), and four female (57%) and two of three partial responders was female and one male. Of the relapsing patients, one was male and one female. These above-mentioned factors were therefore not accurate as prognostic indicators in this group of patients. Table 12 indicates that absolute white cell count as well as absolute lymphocyte count was not a useful prognostic indicator in this group of patients, as two complete responding patients were found to be lymphopenic. One relapsing patient and one partial responder had an increase in both the leukocyte and lymphocyte count. It is unfortunate that the full blood count and differential count were not performed simultaneously with the T-cell subsets. Had all these tests been performed concurrently, absolute T cell numbers could be compared as well as percentages of cells. Parker and co-workers (1994), found that LG-NHL patients with lymphocyte counts below 1 x 10^9/l prior to the commencement of treatment demonstrated worse survival than patients with higher counts. The majority of patients in the study undertaken by Parker and his co-workers, presented with lymphocyte counts between 1-3 x 10^9/l, however patients with lymphocyte counts of greater than 3 x 10^9/l showed similar survival. The present study identified two lymphopenic patients, both of whom demonstrated a complete response. This factor therefore did not serve as a prognostic indicator in this trial.

TABLE 12. ANALYSIS OF DIFFERENT PARAMETERS IN CYCLOPHOSPHAMIDE TREATED PATIENTS

Response	Category	Stage	Age	Sex	Absolute leukocytes Normal 4000-11000	Absolute lymphocytes Normal 1200-4000
CR	C	IV	67	F	7530	ND
CR	B	IV	52	F	8700	1044-
CR	B	IV	69	M	6600	746-
CR	C	IV	65	M	8800	2526
CR	C	IV	63	F	5500	1540
CR	C	IV	55	M	7900	3160
CR	C	IV	59	F	4000	ND
PR	C	IV	39	F	5900	1475
PR	A	IV	46	M	8100	2673
PR	A	IV	64	F	35640+	25304+
Relapse	C	IV	40	F	15800+	11534+
Relapse	C	IV	63	M	6790	2037

\+ indicates results above normal values - indicates results below normal values
ND = test not done CR = complete response
PR = partially response

3.3.2. ANALYSIS OF α-IFN TREATED PATIENTS

Table 13 summarises all patients treated with α-IFN and the different parameters of category according to the Working Formulation, stage, age and sex and their subsequent response. It was possible to adequately assess α-IFN treatment in 8 patients. Thirty-eight percent (3 patients) showed a complete response, fifty percent (4 patients) a partial response and thirteen percent (1 patient) progressive disease. The patient with progressive disease subsequently transformed to a higher grade of NHL. Of the patients that demonstrated a complete response, one patient transformed to a higher grade of NHL and one patient relapsed and died (of their disease) after 156 weeks. Of the patients with a partial response, one patient subsequently relapsed and another patient had a reduction and ultimate discontinuation in the α-IFN treatment, due to toxic side-effects. The side effects consisted of severe lack of concentration. Absolute leukocyte and lymphocyte counts at diagnosis are also included. One α-IFN relapsing patient was found to be both leukopenic and lymphopenic, while the other relapsing and one partially responsive patient were lymphopenic. One α-IFN transforming patient was found to have lymphocytosis. All patients were younger than 60 years of age, except for one relapsing patient, who was 60 years old.

TABLE 13. ANALYSIS OF DIFFERENT PARAMETERS IN α-IFN TREATED PATIENTS

Response	Category	Stage	Age	Sex	Absolute leukocytes Normal 4000-11000	Absolute lymphocytes Normal 1200-4000
CR	Unclassified	IV	40	F	10300	1308
PR	C	III	46	M	6300	2186
PR	C	III	28	F	8200	1263
PR	C	IV	30	M	8600	384-
Relapse	C	IV	51	M	9300	856-
Relapse - died	T-cell	IV	60	F	1400-	434-
Transform	B	IV	57	F	8400	1344
Transform	C	III	50	M	5630	9001+

+ indicates results above normal values - indicates results below normal values
CR = complete response PR = partially response

3.3.3. ANALYSIS OF MISCELLANEOUS TREATED PATIENTS

Table 14 summarises all patients treated off protocol and the different parameters of category according to the Working Formulation, stage, age and sex and their subsequent response. Absolute leukocyte and lymphocyte counts at diagnosis are also included.

TABLE 14. ANALYSIS OF DIFFERENT PARAMETERS IN MISCELLANEOUS TREATED PATIENTS

Response	Category	Stage	Age	Sex	Absolute leukocytes Normal 4000-11000	Absolute lymphocytes Normal 1200-4000
CR	C	IV	58	F	4140	1242
CR	C	I	77	F	6330	1519
CR	C	I	74	M	7690	1307
CR	B	I	54	M	6400	1792
CR	B	IV	37	F	7700	1463
PR	C	IV	40	F	14200+	4970+
MR	A	IV	70	F	3500-	1754
MR - died	C	IV	64	F	7300	1767
PD	C	IV	44	M	4700	1410
PD	B	IV	60	F	12500+	8378+
No change - died	C	IV	64	M	32250+	22253+
Relapse	Unclassified	IV	70	M	7900	2236

+ indicates results above normal values
CR = complete response PR = partially response
MR = minimal response PD = progressive disease

3.3.4. ANALYSIS OF α-IFN TREATED PATIENTS AND THEIR RESPONSE

Using the baseline NKA and NKA + α-IFN and expressing the *in vitro* α-IFN enhancement as percentage α-IFN stimulation (NKA/NKA + α-IFN × 100) in patients treated with α-IFN *in vivo* (Figure 30), there appeared to be an inverse correlation between *in vitro* and *in vivo* α-IFN response. The percentage stimulation was found to be the highest in α-IFN transforming then relapsing and lowest in responding patients. This finding may be useful in predicting an *in vivo* response from an *in vitro* α-IFN response and would disagree with Ozer and co-workers (1983) who report that there was no correlation between clinical response and *in vitro* enhancement of NKA.

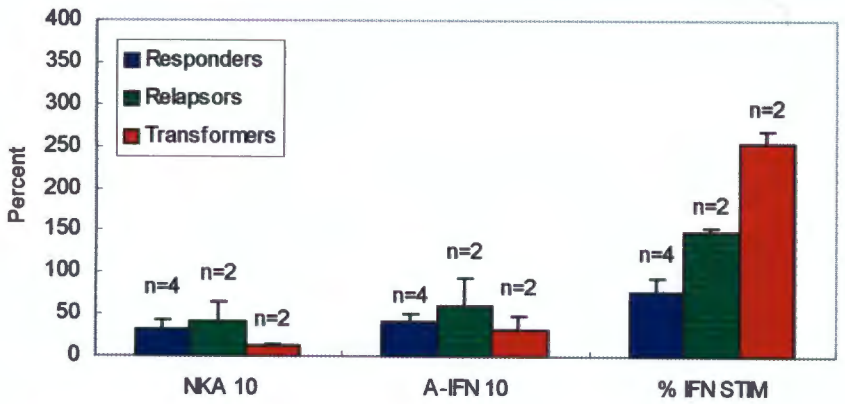


FIGURE 30. A comparison between the NKA, NKA + α-IFN and percentage of stimulation by α-IFN in patients treated with α-IFN *in vivo*.
In an attempt to predict an *in vivo* response to α-IFN, the baseline NKA, NKA + α-IFN and percentage stimulation by α-IFN (NKA/NKA + α-IFN × 100) were compared in all α-IFN treated patients. α-IFN responding patients are referred to as responders and are indicated by blue bars, α-IFN relapsing patients are referred to as relapsors and indicated by green bars and α-IFN transforming patients are called transformers and red bars are used.

3.4.0. MULTIVARIATE DISCRIMINANT ANALYSIS

A multivariate discriminant analysis software program, Statgraphics (STSC Inc., Rockville, MD) was used to analyse all 32 patients in all three different treatment schedules using response as the classification factor. For the purposes of this analysis all patients were either regarded as responding patients (this included complete and partial response) or non responding patients. Patients with progressing disease, patients transforming to a higher grade of NHL, relapsing patients and patients with a minimal response were all regarded as non responders (Czader *et al.*, 1996). Variables for entering into the discriminant function were selected as follows:-A correlation matrix of the immunological parameters at diagnosis was generated and where variables correlated significantly only one (that made the best biological sense) of the set was entered into the discriminant function. If the variable added

"noise" as judged by a decrease in the magnitude of χ^2 then the variable was removed. A linear discriminant function was generated and the magnitude of the standardised coefficients were used to compare the relative importance of the immunological parameters. The best linear discriminant function with unstandardised coefficients was generated and for practical purposes the midpoint between the two centroids was taken as the cutt-off point for predicting responders and non responders. Thus by making use of the generated discriminant function, it is possible to predict with varying degrees of accuracy whether a patient will or will not respond to a particular form of treatment (Afifi and Clark, 1990).

3.4.1. ALL PATIENTS

In the multivariate discriminant analysis, percentages of circulating CD3+, CD4+ and CD8+ were found to be predictive for treatment response when all patients were compared ($p<0.005$). Due to the fact that this is a combination of all the treatment regimens, patients were divided into cyclophosphamide treated patients, α-IFN treated patients and the patients that were treated with other forms of treatment not on the study protocol were kept in a separate group.

3.4.2. CYCLOPHOSPHAMIDE TREATED PATIENTS

In this group of patients, if the percentage of CD3+, CD4+ and CD8+ is compared in a multivariate discriminant analysis, the unstandardized discriminant function coefficients are given for each immunological parameter as follows:-

CD3+ -4.20901

CD4+ 1.67926

CD8+ 5.76613

The constant is 81.6385.

The formula making use of all these coefficients is derived enabling a prediction of response or non-response to cyclophosphamide treatment. For this formula to be significant, p must be < 0.05, in this case p=0.01, therefore this finding is statistically significant.

Formula:-

-(%CD3 X -4.20901) + (%CD4 X 1.67926) + (%CD8 X 5.76613) + 81.6385 = A

If A <11.966, then the patient will respond to cyclophosphamide.

If A > 11.966, then the patient will not respond to cyclophosphamide.

Twelve patients in this study were treated with cyclophosphamide, but in only five patients was the percentages of CD3+, CD4+ and CD8+ ascertained. Although in cyclophosphamide treated patients, the response of all five patients in whom the above-mentioned tests were performed was correctly determined by the use of this formula, it is recommended that this

finding is confirmed in a much larger study. This formula should therefore be used with caution.

3.4.3. α-IFN TREATED PATIENTS

In this group of patients, it was found by the same multivariate discriminant analysis that the percentage of NK and CD3+ cells as well as the ratio of percentage CD4+ to CD8+ cells could be used in predicting the response or non-response of α-IFN. The p value in this case is <0.05.

The formula is as follows:-

-(NK% X 0.88770) - (%CD3 X 1.13259) + (CD4:CD8 X 9.76602) + 72.0362 = B.

If B > 19.41607, then patients will respond to α-IFN.

If B < 19.41607, then patients will not respond to α-IFN.

Eight patients in this study were treated with α-IFN, of which five had the above-mentioned tests performed. In all these five patients, the response was correctly determined. This finding must be interpreted with caution, as it would need further confirmation in a much larger trial.

3.5.0. COMPARISON OF CYCLOPHOSPHAMIDE AND α-IFN TREATED PATIENTS

A Kaplan-Meier curve was generated to compare cyclophosphamide and α-IFN treated patients to ascertain which of the two treatments was more favourable (Figure 31). Time to treatment failure of the two different treatment regimens was compared (Matthews and Farewell, 1985). Treatment failure included both relapse and transformation to a higher grade of NHL. Although cyclophosphamide treatment appeared to be far more favourable than α-IFN treatment, the difference between these two treatments did not quite reach statistical significance (p=0.06).

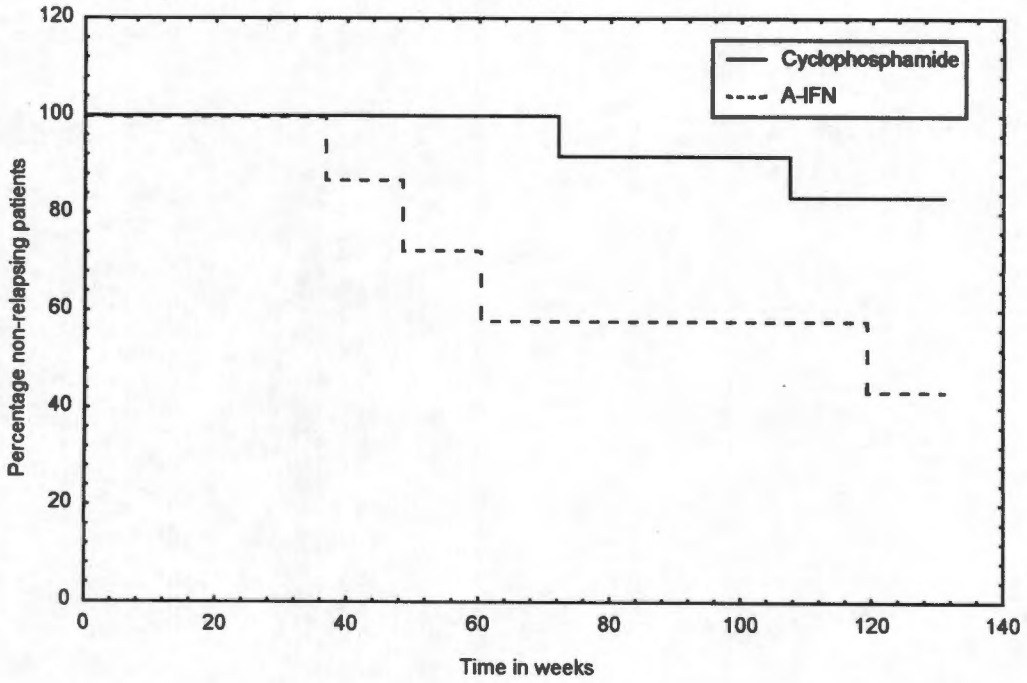


FIGURE 31. Kaplan-Meier curve comparing cyclophosphamide and α-IFN treated patients.

A Kaplan-Meier curve comparing (on the X-axis) time in weeks to treatment failure and on the Y-axis, the percentage of non-relapsing patients. Treatment failure included both relapsing patients and those that transformed to a higher grade of NHL. The difference between cyclophosphamide treatment and α-IFN (A-IFN) treatment did not reach statistical significance (p=0.06).

4. DISCUSSION

Due to the fact that little progress has been made in the treatment of LG-NHL and the ultimate prognosis for these patients has remained the same over the past decades, it is clear that innovative treatment approaches are necessary (Hiddemann and Unterhalt, 1994). A major objective of this trial was to study the ability of α-IFN to increase the duration of remission in LG-NHL patients. Of the patients treated with α-IFN, 38% had a complete response and 50% of patients a partial response, totalling 88%. Only 13% showed progressive disease. These response rates are very encouraging, however 25% of patients who had initially responded to treatment relapsed, of which 13% died and 25% transformed to a higher grade of NHL. A total of 50% of the patients failed this treatment (i.e. 25% relapsed and 25% transformed to a higher grade), although some initially responded. Comparing this trial and other trials in the literature, it can be seen that the responses in this current trial compare favourably to those in previous studies (Merigen *et al.*, 1978; Louie *et al.*, 1981; Horning, 1994-b; Ozer *et al.*, 1983; Foon *et al.*, 1984; Van der Molen *et al.*, 1990; Clark *et al.*, 1989; Ozer *et al.*, 1987; Chisesi *et al.*, 1987; Smalley *et al.*, 1992). The trial conducted by Merigan and co-workers in 1978 using non-recombinant α-IFN, showed a partial response rate of 100%. This data was generated from a very small study consisting of only three patients. The only other study which displayed a comparable response rate was the combination trial by Chisesi and co-workers in 1987 which produced a response of 80%. However, attaining a response is not the difficulty in these patients, it is the maintenance of that response which is the major obstacle. The use of maintenance α-IFN is reported to improve remission duration and hence possibly survival (Rohatiner, 1991). This strategy of treating the patient with chemotherapy, followed by maintenance α-IFN is felt to maximise the effects of α-IFN at a time when the patient has minimal residual disease and biological agents would be expected to be most effective. It is also at the stage following immunosuppressive therapy when the immune restorative effects of α-IFN would be most beneficial. Although α-IFN has an established role and definite therapeutic activity in the management of LG-NHL patients (Crowther, 1991; Clemens, 1988), α-IFN therapy in this study did not appear to be the treatment of choice. The Kaplan-Meier curve indicates that cyclophosphamide treatment is more beneficial to the patient than α-IFN treatment, although the difference between these two groups did not attain statistical significance ($p=0.06$). At 131 weeks, 83.3% of cyclophosphamide patients remained in remission, whereas only 43.3% of α-IFN patients were still in remission. Verification of these findings would require a larger study.

As suggested by Maluish and co-workers (1983), it would be further recommended that in any future α-IFN trials, NKA be monitored serially in these patients. The therapy should be

adjusted to enable *in vitro* NKA to be enhanced. If α-IFN treatment is adjusted to cause NKA enhancement, greater clinical benefit might be demonstrated for LG-NHL patients. It should, however be borne in mind that one of the major difficulties in evaluating α-IFN treatment, is that assessment of cell functions in the peripheral blood may not accurately reflect the immune status of a patient.

Ho and co-workers (1992) report a deficiency in the stimulation of α-IFN by the PBMC's of low and high-grade NHL patients after stimulation. α-IFN treatment *in vivo* might have overcome this defect, but in reviewing patients in this current trial treated with α-IFN and the duration of response, this does not appear to be the case.

The use of the multivariate discriminant analysis in the generation of formulae to predict response to the various forms of treatment, was a particularly exciting and apparently novel finding. α-IFN treatment is extremely expensive and by utilising the flow cytometric analysis of lymphocyte subsets at diagnosis and incorporating these into the formula generated via the multivariate discriminant analysis {-(NK% X 0.88770) - (%CD3 X 1.13259) + (CD4:CD8 X 9.76602) + 72.0362 = B. If B > 19.41607, then patients will respond to α-IFN. If B < 19.41607, then patients will not respond to α-IFN}, it would be possible to identify patients most likely to respond to α-IFN treatment. This would prevent patients being treated with α-IFN who would not respond clinically and would result in enormous cost-saving. It would also identify patients unlikely to respond to cyclophosphamide {-(%CD3 X -4.20901) + (%CD4 X 1.67926) + (%CD8 X 5.76613) + 81.6385 = A. If A <11.966, then the patient will respond to cyclophosphamide. If A > 11.966, then the patient will not respond to cyclophosphamide}, enabling the clinician to institute an alternative form of treatment. This finding is invaluable to both patient and clinician, as appropriate therapy could be instituted at diagnosis, whether it be cyclophosphamide or α-IFN. It would also possibly identify patients who would be highly resistant to either forms of treatment. Patients who are unlikely to respond to either of these forms of therapy may be entered into a research treatment regimen without compromising the outcome of their disease, as they would not have responded to the available therapy. This finding needs confirmation in a larger prospective cross-sectional and longitudinal study.

As a second confirmatory test to establish at diagnosis whether a patient would be likely to respond clinically to α-IFN, a low *in vitro* α-IFN augmented NKA response could be used, as those patients were found to respond to α-IFN *in vivo*. This would further increase the chances of detecting the appropriate treatment for all patients. This study has identified both a possible positive indicator (the formula generated in the multivariate discriminant analysis using the flow cytometric phenotypic analysis) and a negative indicator of α-IFN response (a high *in vitro* α-IFN augmented response) and. It would be highly recommended that these

formulae be used at the commencement of any future trial to distinguish putative responding or non-responding patients to either cyclophosphamide or α-IFN treatment. In a larger trial this would confirm whether these conclusions are valid, and if so would prevent patients undergoing unnecessary treatment to which they would not respond. By making use of both of these factors, it would increase the chances of patients being selected for appropriate forms of treatment and minimising the chances of patients suffering a relapse. A larger comparative α-IFN clinical trial would enable valid conclusions to be drawn on whether or not cyclophosphamide therapy is preferable to α-IFN treatment. Urba and co-workers (1988) recommend that for a meaningful comparison to be made in α-IFN treated patients, not only should a large patient sample be utilised, but lengthy follow-up time periods should be instituted to enable valid interpretation of resulting immune function. In any future trials with α-IFN *in vivo* therapy, the patient group in each treatment schedule should ideally be much larger. This would ensure that there are sufficient numbers of patients in the different response categories for valid conclusions to be drawn. Patients should also be evaluated and followed up for extensive periods of time, as years of follow-up are necessary to accurately assess response to treatment, due to the long natural history of LG-NHL, as well as the continual relapsing and remission pattern.

The statistically significant reduction in the percentage of CD3+ and CD8+ cells and increase in percentage NK cells present in the peripheral blood at diagnosis needs further investigation. The different trends for percentage of NK cells in the peripheral blood between responding patients and non-responding patients also needs to be analysed further. Analysis of NK cell numbers present in enlarged lymph nodes could possibly clarify this matter, as authors differ in their opinion as to NK involvement in lymph nodes. As there is no correlation between patients demonstrating a reduction in the percentage of T-cell subsets and either leukopenia or lymphopenia, the question arises as to what comparative analyses of absolute numbers of these subsets would have shown. It would be highly recommended that absolute numbers rather than subset percentages be determined, in order to reach a more valid conclusion. This option is available now on newer flow cytometry instruments. Alternatively, it is essential to ensure that full blood counts and differential counts as well as phenotypic analyses are conducted simultaneously at each patient visit.

5. CONCLUSION

In this current study much valuable information has been gained which has the potential to accurately predict patients' response to treatment at diagnosis and prior to the commencement of any form of therapy. Furthermore, this study has identified several pitfalls that should be avoided in any future such trial. It would be invaluable therefore, to formulate a trial based on the knowledge that has been gained from this study. Prior to the commencement of this study, a statistician was consulted to ascertain how many patients would need to be enrolled in this trial to ensure valid conclusions. This trial failed to accrue sufficient numbers of patients as advised by the statistician. To overcome this problem, all hospitals treating LG-NHL patients in close enough proximity to make a future study viable, would be notified of the trial and encouraged to participate, making it possible to enrol the maximum amount of patients possible in a multi-centre investigation. All patients would have the diagnosis confirmed by one pathologist who is a recognised expert in the field. At baseline and prior to any form of treatment being instituted, the following tests would be undertaken:- full blood count and differential count; percentages of CD3+, CD4+, CD8+, NK (dual CD56 and CD16 markers) and B cells present in the peripheral blood; NKA; α-IFN enhanced NKA. If sufficient material from the lymph node is available, lymphocyte subsets should also be evaluated in the lymph node. By using the formulae derived from the multivariate discriminant analysis, patients can be selected to receive the appropriate medication to which they would respond. As a further confirmatory test, the *in vitro* α-IFN response can be used to select patients most likely to respond clinically to α-IFN (i.e. a low *in vitro* α-IFN augmented NKA response may be suggestive of an *in vivo* response to α-IFN). All patients that are selected to receive *in vivo* α-IFN treatment should be regularly monitored for NKA and treatment adjusted to ensure that no depression in NKA is observed. At three monthly intervals all the above-mentioned tests should be repeated for at least two years. Patients should be monitored for at least five to ten years, due to the long natural history of the disease as well as the continuous pattern of remission and relapse. Factors which mitigate against successful completion of such a study have been identified and include the lengthy duration, cost, number of patients defaulting and the need for a multi-centre study. These factors would need to be addressed by the funding agencies, hospital committees, and patient support groups.

A major objective of a new study would be to see if conclusions drawn from retrospective analysis would hold true in a prospective randomised study, and to confirm whether the formulae derived from the multivariate discriminant analysis are valid. It would also confirm whether an inverse relationship exists between the *in vitro* α-IFN augmented NKA response and the actual *in vivo* response obtained with α-IFN therapy. Furthermore, such a study

would establish whether cyclophosphamide treatment is preferable to α-IFN treatment in LG-NHL patients. If dual markers for NK (CD56 and CD16) are used to determine percentage NK cells in the peripheral blood, it would be possible to determine whether the most lytic NK subset (CD56+CD16+) or least lytic subset (CD56+CD16-) is increased at diagnosis. If phenotypic evaluation is conducted in the lymph node, conclusions could be drawn as to whether or not lymphocytes have translocated from the bloodstream to the site of pathology. By making use of full blood count and differential counts together with the percentages of circulating lymphocytes, absolute cell numbers can be derived which may be more relevant than percentages of lymphocytes. The findings of the current study indicate that immune parameters may be invaluable as predictors of response and possibly also prognosis in LG-NHL. The results strongly support conducting another clinical trial as a matter of urgency, due to the importance that the outcome would have for both clinician and LG-NHL patient in the treatment of this disease.

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