MARJATTA SINISALO

Responses to Vaccine Antigens in Chronic Lymphocytic Leukemia

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ACADEMIC DISSERTATION
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ACADEMIC DISSERTATION
University of Tampere, Medical School
Tampere University Hospital, Department of Internal Medicine and
Centre for Laboratory Medicine
Finland

Supervised by
Professor Juhani Vilpo
University of Tampere
Janne Aittoniemi, MD, PhD
University of Tampere

Reviewed by
Professor Tapani Ruutu
University of Helsinki
Professor Olli Ruuskanen
University of Turku

Distribution
Bookshop TAJU
P.O. Box 617
33014 University of Tampere
Finland

Tel. +358 3 3551 6055
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To the men I love

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals **I-IV**:

I Sinisalo M, Aittoniemi J, Oivanen P, Käyhty H, Ölander RM and Vilpo J (2001): Response to vaccination against different types of antigens in patients with chronic lymphocytic leukaemia. British Journal of Haematology 114: 107-110.

II Sinisalo M, Aittoniemi J, Käyhty H and Vilpo J (2002): *Haemophilus influenzae* type b (Hib) antibody concentrations and vaccination responses in patients with chronic lymphocytic leukaemia: predicting factors for response. Leukemia & Lymphoma 43: 1967-1969.

III Sinisalo M, Aittoniemi J, Koski T, Tobin G, Thunberg U, Sundström C, Rosenquist R, Käyhty H and Vilpo J. (2004): Similar humoral immunity parameters in chronic lymphocytic leukaemia patients independent of V_H gene mutation status. Leukemia & Lymphoma 45: 2451-2454.

IV Sinisalo M, Vilpo J, Itälä M, Väkeväinen M, Taurio J and Aittoniemi J (2007): Antibody response to 7-valent conjugated pneumococcal vaccine in patients with chronic lymphocytic leukemia.Vaccine 26: 82-87.

ABBREVIATIONS

CLL	chronic lymphocytic leukemia
CLL-U1	CLL-specific gene
CD	"cluster of differentiation" (surface antigen in cell)
CDC	Centers for Disease Control and Prevention
CI	confidence interval
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FISH	fluorescence in situ hybridization
GMC	geometrical mean concentration
Hib	*Haemophilus influenzae* type b
HIV	human immunodeficiency virus
Ig	immunoglobulin
IgHV	immunoglobulin heavy-chain variable
MBL	mannan-binding lectin
M-CLL	mutated CLL
NK	natural killer (cell)
PCR	polymerase chain reaction
TCL-1	the leukemia/lymphoma oncogene
UM-CLL	unmutated CLL
WHO	World Health Organization
ZAP-70	zeta-associated protein 70

ABSTRACT

Background. Chronic lymphocytic leukemia (CLL) is the most common malignant blood disorder in the western world. It is characterized by an accumulation of malignant, mature-appearing but functionally incompetent B-lymphocytes in blood, bone marrow and lymphoid tissues. Patients with CLL are susceptible to infections, this arising from humoral and cell-mediated immunodeficiency associated with CLL itself as well as from treatment of the malignancy.

Infections are the most frequent cause of death in these patients. They are especially prone to infections in the respiratory tract, for example pneumonias and sinusitis. *Streptococcus pneumoniae* is the most prominent single pathogen. In advanced stages of the disease opportunistic pathogens such as herpes viruses and fungi also become more prevalent.

Treatment of CLL does not improve humoral immunity, and many new effective treatments - for example monoclonal antibodies - also disturb cell-mediated immunity. Prophylactic antimicrobials or intravenous immunoglobulins have not solved the problem of infections.

Active immunization by vaccines has effectively eradicated many infections among the healthy population, but their impact in CLL patients has remained unclear. The vaccines mostly used are bacterial polysaccharides, which are, however, weak immunogens, especially when the immune system is compromised. Conjugation of polysaccharides to protein carrier renders them more immunogenic, and they act like T-cell-dependent antigens and also induce an immunological memory.

Aims. In this series we evaluated the specific antibody responses to different types of vaccine antigens in CLL patients, and sought factors predictive of

vaccination-induced antibody responses. Special interest focused on conjugate vaccines, and *Streptococcus pneumoniae,* which is the main pathogen underlying serious infection in patients with CLL.

Subjects and methods. The study cohorts comprised CLL patients from Tampere and Turku University Hospitals. The majority had early-stage disease without a heavy treatment history. The control groups consisted of immunologically healthy persons. The polysaccharide vaccine used in the studies was 23-valent pneumococcal vaccine (Pnu-Immune®), the protein vaccine tetanus toxoid (Tetanus-d-rokote®). The conjugate vaccines were *Haemophilus influenzae* type b (HibTITER®) and 7-valent pneumococcal conjugate vaccine (Prevenar®). We measured concentrations of specific serum antibodies to vaccine antigens before and one month after vaccination.

Results. Vaccination responses to plain polysaccharide pneumococcal antigens were very weak in CLL patients compared to controls, only a few in fact evincing any response at all. In turn, both *Haemophilus influenzae* type b (Hib) and 7-valent pneumococcal conjugate vaccines were more immunogenic in CLL patients. A high proportion of patients reached the concentrations considered protective after vaccination with conjugate vaccines. The response rates were compromised by advanced disease state, chemotherapy and hypogammaglobulinemia. Every fourth CLL patient mounted a significant response to at least 6 pneumococcal antigens after 7-valent pneumococcal conjugate vaccine. Almost 40% of them developed a significant response to at least six pneumococcal antigens out of the seven included in the vaccine if the vaccine had been administered at an early stage in the disease. Similar rates were reached with Hib conjugate vaccine, where one in five CLL patients developed a significant response.

Conclusions. Pneumococcal polysaccharide vaccine did not significantly increase antibody concentrations in CLL patients after vaccination. Pneumococcal and Hib conjugate vaccines, in turn, proved considerably more immunogenic. Conjugate vaccines appeared to be more effective in younger

CLL patients with normal serum immunoglobulin concentrations and less advanced stage of disease. In any case, even many patients with advanced CLL disease and poor prognostic factors such as unmutated CLL responded to both conjugate vaccines. According to the present findings it seems reasonable to vaccinate all CLL patients with conjugate vaccines, and the vaccination should be delivered early in the course of the disease.

TIIVISTELMÄ

Tausta. Krooninen lymfaattinen leukemia (KLL) on yleisin pahanlaatuinen verisairaus länsimaissa. Siinä vereen, luuytimeen ja imukudoksiin kerääntyy pahanlaatuisia, kypsän näköisiä, mutta toiminnaltaan epäkypsiä B-lymfosyyttejä. Potilaat saavat herkästi infektioita. Infektioherkkyys johtuu tautiin liittyvästä humoraalisen ja soluvälitteisen immuniteetin häiriöstä. Myös taudin hoito heikentää vastustuskykyä.

Infektiot ovat merkittävin KLL-potilaiden kuolinsyy. Potilaat saavat erityisen herkästi hengitystieinfektioita, kuten keuhkokuumeita (pneumonioita) ja nenän sivuonteloiden tulehduksia (sinuiitteja). Näitä infektioita esiintyy jo KLL:n varhaisvaiheessa. *Streptococcus pneumoniae* (pneumokokki) on merkittävin yksittäinen taudinaiheuttaja. Taudin myöhäisemmässä vaiheessa myös soluvälitteisen immuniteetin häiriöt lisääntyvät ja opportunistiset taudinaiheuttajat, kuten herpesvirukset ja sienet, tulevat yleisiksi.

Perustaudin hoito ei korjaa humoraalista immuniteettia, ja monet uudet tehokkaat hoitomuodot – kuten monoklonaaliset vasta-aineet – häiritsevät myös soluvälitteistä immuniteettia. Infektioita ennaltaehkäisevä mikrobilääkitys tai suonensisäisesti annettu immunoglobuliinihoito ei ole ratkaissut tätä infektio-ongelmaa.

Aktiivinen immunisaatio rokotuksilla on osoittautunut tehokkaaksi terveen väestön infektiosairauksien hävittämisessä, mutta sen teho KLL-potilailla on epäselvää. Yleisimmin käytettyjä rokotteita ovat bakteeripolysakkaridit. Ne ovat kuitenkin heikkoja immunogeenejä, jos immuunisysteemi on häiriintynyt. Kun polysakkaridiantigeenit liitetään kuljettajaproteiiniin, ne muuttuvat immunogeenisemmiksi ja toimivat kuten T-soluista riippuvaiset antigeenit ja kehittävät myös immunologisen muistin.

Tavoite. Tässä tutkimuksessa selvitimme KLL-potilaiden spesifejä vasta-ainereaktioita erityyppisille rokoteantigeeneille. Selvitimme tekijöitä, jotka ennustivat rokotteen aiheuttamaa vastetta. Erityisen mielenkiinnon kohteena olivat konjugaattirokotteet ja *Streptococcus pneumoniae*, joka on tärkein vakavien infektioiden aiheuttaja KLL-potilailla.

Potilaat ja kontrollit. Potilaina oli KLL:aa sairastavia henkilöitä Tampereen ja Turun Yliopistollisista sairaaloista. Suurimmalla osalla oli varhaisvaiheen KLL, eivätkä he olleet saaneet voimakkaita hoitoja. Vertailuryhmän muodostivat immunologialtaan terveet henkilöt. Polysakkaridirokotteena tutkimuksessa oli 23 serotyypin pneumokokkirokote (Pnu-Immune®), ja proteiinirokote oli tetanustoksoidi (Tetanus-d-rokote®). Konjugaattirokotteet olivat *Haemophilus influenzae* tyyppi b (HibTITER®) ja 7 serotyypin pneumokokkikonjugaattirokote (Prevenar®). Määritimme spesifiset seerumin vasta-aineet rokoteantigeeneille ennen rokotusta ja kuukausi rokotuksen jälkeen.

Tulokset. Pneumokokkipolysakkaridirokotteen aiheuttama nousu vasta-aineissa jäi suurimmalla osalla KLL-potilaista olemattoman heikoksi kontrollihenkilöihin verrattuna. Ainoastaan muutamalla yksittäisellä potilaalla oli mitattavia vasteita kuukausi rokotuksen jälkeen. Sen sijaan molemmat konjugaattirokotteet, *Haemophilus influenzae* tyyppi b (Hib) ja 7 serotyypin pneumokokkirokote, olivat immunogeenisiä KLL-potilailla. Suuri osa KLL-potilaista saavutti rokotuksella vasta-ainetason, jota pidetään suojaavana. Vastetta heikensivät pitkälle edennyt KLL, solunsalpaajahoito ja hypogammaglobulinemia. Joka neljäs KLL-potilas saavutti merkittävän vasteen vähintään kuudelle rokotteen sisältämistä seitsemästä pneumokokin serotyypistä rokotuksen jälkeen. Lähes 40% KLL-potilaista reagoi vähintään kuuteen rokotteen sisältämistä seitsemästä pneumokokin serotyypistä merkittävällä vasteella, jos pneumokokkikonjugaattirokote annettiin taudin varhaisessa vaiheessa. Hib-konjugaattirokotteella joka viides potilas saavutti merkittävän vasteen.

Loppupäätelmät. Pneumokokkipolysakkaridirokote ei nostanut merkittävästi KLL-potilaiden vasta-ainetasoja rokotuksen jälkeen. Sen sijaan pneumokokki- ja

Hib-konjugaattirokotteet olivat selvästi immunogeenisempiä KLL-potilailla. Konjugaattirokotteet olivat tehokkaampia nuoremmilla KLL-potilailla, joilla oli normaalit veren immunoglobuliinitasot ja varhaisemman vaiheen tauti. Kuitenkin myös monet potilaat, joilla oli pitkälle edennyt tauti ja huonon ennusteen tekijöitä, kuten mutatoitumaton tautimuoto, reagoivat konjugaattirokotteeseen. Tutkimustulokset viittaavat siihen, että kaikki KLL-potilaat tulisi rokottaa konjugaattirokotteilla ja että rokotukset pitäisi tehdä taudin alkuvaiheessa.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in western countries. It is characterized by an accumulation of small, mature-appearing but functionally incompetent monoclonal long-lived B-lymphocytes in blood, bone marrow and lymphoid tissues (Matutes et al. 1994). Due to a slow disease course it was formerly called “an incurable old man's disease”, and elderly patients were expected to die with CLL rather than from it (Kipps 2007). However, the clinical course of the disease is markedly heterogeneous, the survival of patients ranging from one month to more than twenty years (Rozman and Montserrat 1995). Survival is related to the clinical stage at diagnosis, genetic markers, and the rate of disease progression and to differences in clinical response to chemotherapy.

Infectious complications are the major cause of mortality in patients with CLL. It has been estimated that up to 50% of them suffer from recurrent infections (Molica 1994, Morrison 1998, Tsiodras et al. 2000), and infections are the cause of death in 30% to 60% of cases (Hansen 1973, Itälä et al. 1992, Molica 1994, Rozman and Montserrat 1995, Francis et al. 2006). Susceptibility to infections arises from immunodeficiency associated with CLL as well as with treatment of the malignancy.

The pathogenesis of immunodeficiency in CLL is complex and multifactorial. Humoral defects are the most significant, especially in early-stage disease. Patients also have defects in T-cell, natural killer (NK) cell, neutrophil and complement functions. Furthermore, the cytokine pattern (Kiaii et al. 2005) and antigen presentation (Orsini et al. 2003) are abnormal. Patients with CLL are particularly susceptible to infections caused by capsular pathogens, e.g. *Streptococcus pneumoniae* (Travade et al. 1986, Itälä et al. 1992), which may also be detrimental to patients with hypogammaglobulinemia without CLL and to

splenectomized patients. The systemic infections caused by these pathogens are often lethal. In a retrospective study made by Ahmed and colleagues (2003) in the United States, 40% of CLL patients admitted to hospital with pneumonia died from it. The spectrum of infections has changed over the past two decades in a more opportunistic direction due to therapies with purine analogues and monoclonal antibodies (Wadhwa and Morrison 2006).

Patients with CLL have been vaccinated against influenza virus and *Streptococcus pneumoniae* in many centers, but the effect of vaccination has remained unexplored. In some earlier studies patients with CLL have responded with weak antibody formation to vaccines (Shaw et al. 1960, Cone and Uhr 1964, Jacobson et al. 1988, Mellemgaard et al. 1993, Jurlander et al. 1995).

The purpose of this present study series was to evaluate humoral immunity and antibody responses to different vaccine antigens in patients with CLL. The main focus was on pneumococcal vaccinations, in order to pave the way for clinical prophylaxis for this detrimental infection in CLL.

REVIEW OF THE LITERATURE

1.1. Chronic lymphocytic leukemia (CLL)

1.1.1 Definition and diagnosis

One of the first detailed definitions of chronic lymphatic leukemia was presented by Felix Pinkus in “Diseases of Blood” in “Nothnagel`s Encyclopedia of Practical Medicine” (Pinkus 1905). Leukemia was divided into three types: acute lymphatic, chronic lymphatic and myelogenous leukemia. An increase in lymphocytes in the blood, and enlargement of the lymph glands and the spleen were requisite for a diagnosis of chronic lymphatic leukemia, and the lymphocytes were to be the same size as, or smaller than, the red blood corpuscles (Pinkus 1905). According to the current World Health Organization (WHO) classification, CLL belongs to the mature B-cell neoplasms (Harris 2001). It is characterized by an accumulation of small, mature-like but immunologically incompetent lymphocytes in bone marrow, blood, lymph nodes, spleen and liver, only exceptionally in other tissues. Greater than 30% infiltration of lymphocytes in the bone marrow is consistent with a diagnosis of CLL (Dameshek 1967, Cheson et al. 1996). According to National Cancer Institute-Sponsored Working Group guidelines for the diagnosis of CLL, the peripheral blood should exhibit an increase in the number of small, mature-appearing lymphocytes to more than 5 x 10^9/l for the duration of at least 3 months (Cheson et al. 1996). Flow cytometry is nowadays essential in confirming the clonality of the B-cells and ruling out other morphologically similar lymphoid malignancies.

1.1.2. Occurrence

Among the leukemias, CLL is the most common type in Europe and the United States. Of all mature B- and NK-cell lymphomas it comprises about 7% (Harris 2001). In Finland about 180 new cases are diagnosed every year, 60% of them men and 40% women. During the last 50 years, no increase in incidence has been noted (Finnish Cancer Registry 2007). For unknown reasons CLL is uncommon in the Far East, where chronic T-cell leukemias are more common (Tamura et al. 2001). The disease is diagnosed mostly in individuals in the fifth and sixth decades of life, and sometimes exhibits an indolent clinical course (Rai and Patel 1995). It has more aggressive features in men than in women (Molica 2006). Asymptomatic CLL cases have doubled from 30% to 60% of all cases during recent decades due to the increased number of blood tests performed for other medical or surgical reasons (Kalil and Cheson 1999).

1.1.3 Etiology

The etiology of CLL is unknown (Rozman and Montserrat 1995). There is evidence of a genetic component. The risk of CLL among first-degree relatives is increased about sevenfold (Goldin et al. 2004), and familial CLL cases have an earlier age at diagnosis than sporadic cases (Cuttner 1992, Catovsky 1997). Recently, certain genetic abnormalities have been linked to the pathogenesis of inherited CLL (Ng et al. 2007).

Infection - or some irritation of the lymphatic tissue - was already envisaged as an etiologic agent for chronic lymphatic type of leukemia a hundred years ago (Pinkus 1905). In a recent Danish population-based study, a personal history of pneumonia was associated with a significantly increased risk of CLL (Landgren et al. 2007). Lower respiratory tract infectious agents causing pneumonia might be potential triggers for CLL development, or pneumonia could be a consequence of an immune disorder called monoclonal lymphocytosis of undetermined significance (MLUS) preceding CLL. Evidence is accruing to suggest that antigen stimulation (auto- or foreign antigen) plays a key role in the

development and progression of CLL (Messmer et al. 2004, Tobin et al. 2004, Ghia et al. 2005, Landgren et al. 2006, Stamatopoulos et al. 2007).

1.1.4 The CLL cell

CLL cells are small, mature-like lymphocytes which are functionally incompetent. In flow cytometry, the CLL cell is typically CD5, CD23 and CD19 positive, evinces weak expression of CD20 and surface immunoglobulin, and it is FMC7 and CD22 negative (Matutes et al. 1994). Typical B-cell markers, CD19 and CD20, together with surface immunoglobulin (albeit only about 10% as much as in polyclonal B-cells) make it a B-cell. The presence of CD5 on CLL cells is unexpected, as it is expressed on all mature T-cells but normally on only a minor subpopulation of B-cells. CD23 is usually undetectable in normal B-cells, but present in CLL B-cells.

CLL has long been regarded only as a disease of accumulation due to a presumed defect in the programmed cell death called apoptosis. Recent data, however, suggest that B-cells are also born at an accelerated rate, the rate of proliferation varying among patients (Chiorazzi et al. 2005, Messmer et al. 2005). More than 99% of CLL cells are in the resting (G0/early G1) phase of the cell cycle (Caligaris-Cappio and Hamblin 1999).

The B-cell receptor, whose main component is the immunoglobulin (Ig) molecule, is essential to the B-cell. The B-lymphocyte becomes committed to produce a particular antibody by rearrangements of the Ig genes V, D and J. When the B-cell receptor reacts with an antigen in the germinal centre of the lymph node, each time it divides it creates a subtle variation in the B-cell receptor to make the antibody fit in the best possible way for the antigen. These small point alterations are called somatic mutations. There was much debate in the 1990s as to whether the CLL cell resembled a pre-germinal or a post-germinal centre cell. It is now proven that there are two types of CLL cells, and also two types of the disease with very different prognosis: 50-70% of CLL patients have somatic mutations in immunoglobulin heavy-chain variable (IgHV) genes, as if they had matured in a lymphoid follicle and function as memory B-lymphocytes (post-germinal), whereas the other types are naive, mantle zone-like

cells (pre-germinal) (Damle et al. 1999, Hamblin et al. 1999). However, gene expression data indicate that both unmutated and mutated CLL cells have a memory B-cell origin (Klein et al. 2001), and a growing body of evidence suggests that the CLL cell must be an antigen-experienced B-cell (Caligaris-Cappio and Ghia 2007).

1.1.5 Clinical features

The course of CLL disease can vary significantly, some patients surviving for many years with minimal or no treatment, others succumbing rapidly - even within months - despite treatment. The majority of patients are revealed because of lymphocytosis of at least 5 x 10^9/l in an incidental blood count (Kalil and Cheson 1999). The disease is staged according to the presence of lymphadenopathy and/or splenomegaly and features of bone marrow suppression. Most patients are in an early stage of the disease at presentation, and in perhaps 30% of them CLL will never progress. This group has a normal life expectancy and requires no treatment beyond reassurance (Eichhorst and Hallek 2007). Progression involves an increasing white cell count, enlarging lymph nodes and spleen, anemia and thrombocytopenia. Autoimmune complications are common in CLL, occurring in up to a quarter of all patients during the course of the illness. The most common manifestation is autoimmune hemolytic anemia (AIHA), followed by immune thrombocytopenia (ITP) (Hamblin 2006). Autoimmune episodes may be triggered by treatment, particularly with purine analogues (Borthakur et al. 2007). The development of a more aggressive, diffuse large-cell lymphoma (Richter's transformation) occurs in about 5% of CLL patients (Giles et al. 1998). Secondary malignancies are notably common. In a recent study of 2083 patients with CLL, other cancers were noted in approximately every fourth patient in a median follow-up of about 6 years. Patients who had received treatment for CLL had similar rates of other cancers compared to untreated patients (Tsimberidou et al. 2006).

1.1.6 Cytogenetics

Genetic analyses by fluorescence in situ hybridization (FISH) and DNA sequencing have greatly improved our understanding of pathogenic events and prognostic markers in CLL. By these methods, genomic aberrations are detected in over 80% of CLL cases. There is no single specific cytogenetic abnormality in the condition. Genetic subgroups with distinct clinical features have been identified.

Several genetic aberrations have proved useful prognostic markers in CLL, some of them possibly also having an etiological role in disease progression. The 17p deletions, reflecting tumor suppressor gene p53 abnormalities, are described as the strongest independent predictors for aggressive disease, resistance to chemotherapy and early death (Oscier et al. 2002, Grever et al. 2007). In contrast, isolated deletions at chromosome 13q14, which are associated with heavy-chain somatic mutations and a normal karyotype, are associated with longer survival. The 11q deletions, which point to ataxia telangiectasia-mutated protein (ATM) defects, are associated with marked lymphadenopathy and rapid disease progression (Dohner et al. 1997). Trisomy of chromosome 12 is often associated with unmutated IgHV genes, atypical cellular morphology and progressive disease (Caligaris-Cappio and Hamblin 1999).

The somatic hypermutation status of IgHV genes can divide CLL into two prognostic subsets. If the CLL cells have IgHV genes with ≥98% sequence homology with the nearest germ line gene, they are considered unmutated (UM-CLL). If the variation percentage is >2%, the cells are considered mutated (M-CLL) (Tobin et al. 2005). Recently, two new candidate genes, the leukemia/lymphoma oncogene (TCL-1) and the CLL-specific gene (CLL-U1) have been found to be overexpressed in CLL (Herling et al. 2006, Josefsson et al. 2007).

1.1.7 Staging and prognostic factors

For the past 30 years CLL has been staged by one of two systems devised by two researchers in the field, Kanti Rai from the United States and Jacques-Louis

Binet from France (Rai et al. 1975, Binet et al. 1981). Both give an estimate of how advanced the disease is, based on simple clinical and laboratory measurements commonly available. These systems have a good correlation with survival (Figure 1).

According to the United States National Cancer Institute, unfavorable prognostic factors independent of clinical stage have been: age over 55 years, male sex, black race and poor performance status. There have been no differences between older and younger patients in presenting features, response to therapy or duration of response (Kalil and Cheson 1999). In recent years, many genetic and laboratory markers have been presented as new candidates for prognosis and response prediction. Serum and cellular markers which may predict outcome include: lymphocyte doubling time, beta-2-microglobulin, lactate dehydrogenase, thymidine kinase, lipoprotein lipase, thrombopoietin, soluble CD23, surface CD38, and intracellular zeta-associated protein 70 (ZAP-70) (Crespo et al. 2003, Orchard et al. 2004, Koller et al. 2006).

Patients with M-CLL show superior survival compared to UM-CLL cases, the former having an average survival of 25 years, the latter only 8 years (Damle et al. 1999, Hamblin et al. 1999, Chiorazzi et al. 2005). Exceptions in M-CLL are mutations in the IgHV3-21 region, which reflect poor prognosis (Tobin et al. 2003). Currently, measurement of IgHV mutation status is very laborious and expensive, and cannot be used in daily routine.

The expression of the tyrosine kinase, ZAP-70, in CLL seems to correlate with the mutational status of the IgHV, the clinical course and patient prognosis in large patient series (Crespo et al. 2003, Orchard et al. 2004, Rassenti et al. 2004). UM-CLL cells express ZAP-70 genes, and intracellular ZAP-70 increases. This has been an even stronger predictor of disease progression than the mutational status of IgHV (Rassenti et al. 2004). The test is readily available, but its use needs to be harmonized for standard application.

Some genetic markers, for example deletions in chromosomes 17p and 11q reflect poor prognosis despite the mutational status of IgHV. Some of these markers, detected by FISH, are used routinely as prognostic factors in addition to the traditional staging systems (Binet et al. 2006).

Also telomere length and telomerase activity, TCL-1 and CLL-U1 expressions, and micro RNAs are associated with prognosis (Hultdin et al. 2003, Calin et al. 2005).

Figure 1. CLL staging and survival according to Binet and Rai

Binet classification		median survival (years)
A	no anemia or thrombocytopenia and fewer than 3 areas of lymphoid involvement	12
B	no anemia or thrombocytopenia with 3 or more areas of lymphoid involvement	5
C	anemia (Hb <100 g/l) and/or thrombocytopenia (platelets <100 x 10^9/l) regardless of the number of areas of lymphoid enlargement	2

Binet et al. 1981

Rai staging system		median survival (years)
0	Lymphocytosis only	>15
I	Lymphocytosis with lymphadenopathy	9
II	Lymphocytosis with hepatomegaly or splenomegaly	5
III	Lymphocytosis with anemia (Hb <110g/l)	2
IV	Lymphocytosis with thrombocytopenia (platelets <100 x 10^9/l)	2

Rai et al. 1975

1.1.8 Treatment and outcome

CLL is a disease requiring no treatment in an asymptomatic stage. The overall median survival is more than 5 years, and the presence of anemia and thrombocytopenia adversely affects prognosis (Tefferi and Phyliky 1992). Approximately 30% of CLL patients never need treatment. For them “watch and wait” is the best strategy, since early treatment has no positive impact on survival (Cheson et al. 1996). Difficulties arise from the heterogeneity of the disease; it is difficult to identify patients who will benefit from more aggressive therapy. Some patients can achieve durable complete response and even molecular remission with newer treatment regimens, and the goal of therapy may in the future change from palliation to a potential cure.

A patient in an early stage (Binet A and B without symptoms) - even with poor genetic prognostic profiles - should not be treated outside clinical trials. Advanced disease stages (Binet stage C and symptomatic B) indicate treatment (Eichhorst and Hallek 2007). Patients in good physical condition should be offered combination therapies with chemotherapeutic agents such as fludarabine and cyclophosphamide, possible with monoclonal antibody targeted to CD20 (rituximab). Patients with impaired physical condition or co-morbidity may be offered chemotherapeutic agents such as chlorambucil or dose-reduced fludarabine monotherapy for symptom control. Those with symptomatic disease and chromosome 17p deletions respond poorly to fludarabine and cyclophosphamide, and for them the first-line therapy could be monoclonal antibody against CD52 (alemtuzumab) or/and methylprednisolone. For younger patients with matched stem cell donor, allogeneic stem cell transplantation might prove curative. Autologous transplantation, which is not a curative treatment, might be considered in selected patients in randomized trials (Jantunen et al. 2006). Currently, the management of CLL is in a phase of rapid evolution, and risk stratification using prognostic scores may increasingly guide treatment in the future.

1.2. Immunodeficiency and infections in CLL

1.2.1 Immunodeficiency

CLL is associated with immunological defects, the nature of which is complex and multifactorial. They involve factors related both to leukemia itself and to immunosuppressive treatment (Molica 1994). The problem precedes treatment and the development of hypogammaglobulinemia (i.e. low serum IgG), and it emerges long before the lymphoid system is overwhelmed by infiltration with CLL cells. Both adaptive (specific) and innate (non-specific) components are known to be involved.

Adaptive immunity has been compromised in many cases of CLL (Kay and Perri 1988, Zaknoen and Kay 1990, Caligaris-Cappio and Hamblin 1999). The association between hypogammaglobulinemia and infections in CLL patients has been recognized since 1960 (Hudson and Wilson 1960). On the other hand, conflicting results have also been published (Hansen 1973). Hypogammaglobulinemic CLL patients have evinced a typical pattern of infections, being particularly vulnerable to recurrent upper respiratory tract infections caused by encapsulated organisms like *Streptococcus pneumoniae* and *Haemophilus influenzae*. In these patients, the specific antibody concentrations against pneumococcal polysaccharide antigens have also been low (Chapel and Bunch 1987). The incidence and severity of hypogammaglobulinemia is observed to be increased with advanced disease stage (Molica et al. 1993). Hypogammaglobulinemia has meant in most studies serum IgG concentrations below the normal range, and as high as 8 g/l has been the lowest limit in some studies (Shaw et al. 1960). However, in patients with common variable immunodeficiency (CVID), the serum concentrations of IgG and IgA are more than 2 standard deviations below the age-matched normal values, usually less than 3 g/l (Kainulainen et al. 2001). In patients with CLL, clinically significant hypogammaglobulinemia probably manifests with higher values, since they have many additional factors suppressing immunity.

Of the other Ig isotypes, IgA deficiency has been correlated with poorer survival (Rozman et al. 1988), and a decreased serum concentration of IgA has also been shown to be an independent risk factor for infection (Aittoniemi et al. 1999).

Although T-cell levels have usually been normal in untreated CLL patients, functional defects have been observed in CD4 (helper) cells, and CD8 (cytotoxic) cell activity has been increased, leading to a reversal of the CD4/CD8 ratio, which may correlate with disease stage and degree of hypogammaglobulinemia (Chiorazzi et al. 1979, Catovsky et al. 1981, Kay 1981, Platsoucas et al. 1982). A decrease in NK-cell activity has been reported (Ziegler et al. 1981), as well as an abnormal cytokine pattern in CLL cells (Kiaii et al. 2005).

Patients with CLL have also exhibited defects in antigen presentation. The most important antigen-presenting cells are dendritic cells. In CLL, circulating dendritic cells have been unable to stimulate an effective T-cell response, possibly due to interfering cytokines secreted by the CLL cells (Rezvany et al. 2000, Messmer et al. 2004).

The complement system plays a critical role in innate immunity by opsonizing encapsulated organisms and activating neutrophils. Defects in complement proteins, mostly in properdin, have been demonstrated in CLL patients (Schlesinger et al. 1996). A correlation has been noted between low initial complement levels and decreased survival (Varga et al. 1995). Mannan-binding lectin (MBL), which is the first component in the complement lectin pathway and an acute-phase reactant, is an important factor in opsonization of microbes. Its concentrations have been detected to be higher in CLL patients with infections compared to those without (Aittoniemi et al. 1999).

Toll-like receptors are the regulators of innate immunity in detecting pathogens and initiating adaptive immunity by stimulating T-cell proliferation. Their expression and functions have been similar in CLL cells and normal B-cells (Grandjenette et al. 2007).

The absolute neutrophil count is often normal in untreated CLL patients, and neutropenia is mostly a consequence of myelosuppressive therapy. Some defects in neutrophil enzymes (myeloperoxidase and lysozyme) and also in

neutrophil functions (migration and chemotaxis) have been detected in CLL patients with active infection (Itälä et al. 1996).

1.2.2 Infections

Infections have for many decades been the major cause of morbidity and mortality in patients with lymphoproliferative disorders and CLL (Ultman et al. 1959, Boggs et al. 1966, Hansen 1973, Itälä et al. 1992, Molica 1994, Rozman and Montserrat 1995, Tsiodras 2000). It has been estimated that up to 50% of CLL patients will die due to infection (Molica 1994). A recent retrospective study has revealed that 36% had had at least one major infection (requiring hospital admission and intravenous antimicrobials) in the preceding 5-6 years (Francis et al. 2006).

Infections have affected mainly the respiratory tract. Other common sites have been the urinary tract, skin and soft tissues, and the bloodstream in severely neutropenic CLL patients (Francis et al. 2006). A great majority (>80%) of documented infections have been bacterial (Francis et al. 2006), *Streptococcus pneumoniae* being one of the most prominent single pathogens (Itälä et al. 1992, Francis et al. 2006). According to one study, pneumococcal sepsis was a greater problem in patients with lymphoproliferative disorders than in splenectomized patients in the United States over a follow-up period of 1 ½ years, and mortality in these patients was also higher (Gowda et al.1995). Among CLL patients admitted to hospital due to respiratory tract infection, pneumonia has been the final diagnosis in 75% of cases, with 40% mortality (Ahmed et al. 2003).

Treatment of CLL with purine analogues and monoclonal antibodies such as rituximab and alemtuzumab leads to profound and sustained T-cell immunodeficiency. As a result of this, the spectrum of pathogens has altered from common bacteria towards more opportunistic pathogens such as *Pneumocystis jirovecii*, *Listeria monocytogenes*, mycobacteria, herpes viruses and *Candida* species (Anaissie et al. 1998). Reactivation of herpes viruses has become a common problem in patients with CLL.

Multiple factors have been associated with the number and severity of major infections in CLL patients: low hemoglobin concentration, the number of

previous chemotherapy regimens, low immunoglobulin concentrations (IgG, IgA and IgM), low concentrations of specific pneumococcal polysaccharide antibodies, disease stage B or C, positive CD38 expression, unmutated IgHV, and abnormal genetics (Chapel and Bunch 1987, Aittoniemi et al. 1999, Hensel et al. 2003, Francis et al. 2006). Mortality related to infections has been associated with older age, disease status, positive CD38, IgHV status and genetic abnormalities (Francis et al. 2006). Disease activity and the extent of pretreatment have had a stronger impact on the risk of severe infectious complications than hypogammaglobulinemia in CLL patients (Hensel et al. 2003).

1.2.3 Prevention of infections

Attempts to control immunodeficiency in CLL have been limited. Since very little can be done to reinforce the host's innate immune system, all efforts to enhance the patient´s own adaptive immunity are important in CLL.

The natural means of preventing infections in CLL would be successful chemo- or radiotherapy for the leukemia itself, which would hopefully result in the recovery of the immune system. In clinical practice, fewer infections are seen in good-response patients, although hypogammaglobulinemia rarely disappears (Ultman et al. 1959, Rai and Montserrat 1987).

Numerous investigations into possible preventive means have been undertaken using intravenous immunoglobulin. In some studies even serious infection rates have been reduced (Griffiths et al. 1989, Jurlander et al. 1994, Molica et al. 1996), while in others no effect has been observed (Cooperative Group for the Study of Immunoglobulin in Chronic Lymphocytic Leukemia 1988, Chapel et al. 1994). No effect on mortality has been observed, and the cost-effectiveness of this treatment has been considered unacceptable (Weeks et al. 1991). In clinical practice, CLL patients with recurrent sinus and pulmonary infections and low IgG concentrations are treated with intravenous immunoglobulin for at least 6 months to evaluate the effect of this treatment in the prevention of infections. On the other hand, new therapies - for example purine analogues and monoclonal antibodies - lead to secondary T-cell defects

and opportunistic infections which are not likely to be preventable by intravenous immunoglobulin therapy.

Antimicrobial prophylaxis has been used in clinical practice, but no controlled trials of its efficacy have been reported. No prospective studies have been made comparing intravenous immunoglobulin treatment to prophylactic antimicrobials. Patients with advanced disease stage, a heavy treatment history and elevated serum creatinine might need prophylactic antimicrobials, especially in the case of purine analogue and monoclonal antibody therapies (Anaissie et al. 1998, Morrison 2001).

Vaccines are, in general, a well-established means of preventing infections. Nonetheless, very little is known as to their efficacy in CLL patients, and no consensus prevails regarding their use in these patients.

1.3. Vaccines

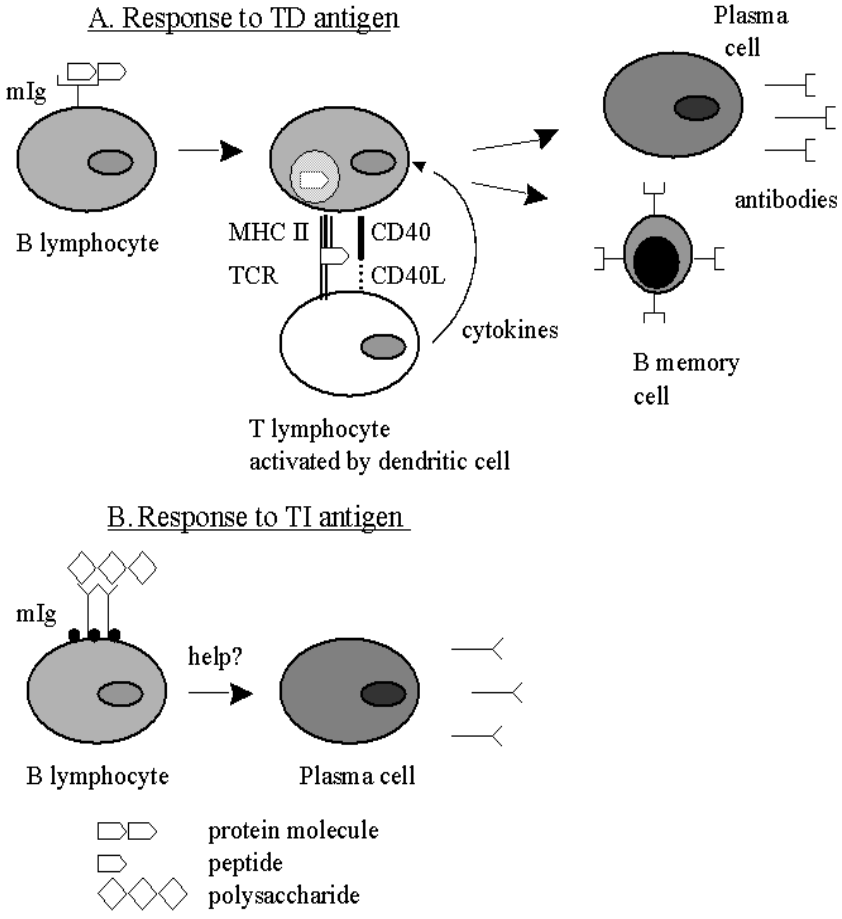
1.3.1 Vaccine types

Population-based vaccine programs have effectively eradicated many infectious diseases and new vaccines are continuously being developed. Three types of vaccines are in current use: live attenuated microorganisms, inactivated microorganisms and purified macromolecules (Goldsby et al. 2000). The latter include polysaccharide and different protein vaccines. The immunogenicity of vaccines is improved by adjuvants, which act via receptors in the innate immune system, and many of them appear to be ligands for Toll-like receptors (Hauguel and Hackett 2008).

Polysaccharides are the most widely used bacterial components for vaccines. Nevertheless, polysaccharide antigens are weak immunogens, especially if the immune system is immature or suppressed. Polysaccharide antigens induce a response which is T-cell-independent (Käyhty et al. 1984). No immunologic memory is induced, and as a result no boosted response is seen on repeated injections.

The modification of polysaccharide antigens to a T-cell-dependent, immunologic memory-inducing form by conjugating them to a protein carrier has rendered the bacterial polysaccharide antigens more immunogenic (Goldblatt 2000). Conjugation to a protein carrier leads to activation and clonal expansion of carrier-specific T-cells, as well as polysaccharide-specific B-cells (Stein 1992). T-cell-dependent and T-cell-independent vaccine responses are illustrated in Figure 2. Conjugated polysaccharide Hib vaccines were first licensed in 1987 and proved effective in infants; they induced higher antibody titers than unconjugated vaccines (Eskola and Käyhty 1998). The use of conjugated Hib vaccine caused a significant decline in invasive Hib infections in infants in the developed world (Murphy et al. 1993, Eskola and Käyhty 1996, Peltola 2000).

Figure 2. Responses to T-cell-dependent (TD) and T-cell-independent (TI) antigens (with permission from Väkeväinen M. 2000, Dissertation, University of Helsinki)



1.3.2 Pneumococcal vaccines

1.3.2.1 Polysaccharide vaccine

Many European countries and the United States have recommended vaccination of all elderly individuals over 65 years and also immunocompromised patients with pneumococcal polysaccharide 23-valent vaccine (CDC 2006). However, there is controversy as to vaccine efficacy in these patient groups (Simberkoff et al. 1986, Forrester et al. 1987, Örtqvist et al. 1998, Watson 2002). In a meta-analysis of 14 randomized trials involving 49 000 individuals, pneumococcal polysaccharide vaccine prevented definite pneumococcal pneumonia by 71%, presumptive pneumococcal pneumonia by 40% and mortality due to pneumonia by 32%, but not all-cause pneumonia or death. No preventive effect was seen in the subgroup of patients aged 55 years or more, possibly due to a lack of statistical power (Cornu et al. 2001). Another meta-analysis of nine trials demonstrated significant protection in non-immunosuppressed subjects, but no effect in high-risk cases (Fine et al. 1994). In a study by Butler and associates the overall efficacy in preventing serious infection caused by serotypes included in the vaccine was 57%. Efficacy for immunocompetent persons older than 65 years was 75% and for high-risk patients 49%. However, efficacy was not documented for example among patients with lymphoma, leukemia or multiple myeloma, sample sizes for these groups being unfortunately small (Butler et al. 1993).

Pneumococcal polysaccharide vaccine efficacy also depends on serotype coverage, and on how the immunogenicity of the each serotype varies (Poland 2001). The serotype-specific antibody concentrations decline after 5-10 years, and re-immunization is recommended at 5-year intervals in the case of immunosuppressed patients. Polysaccharide vaccine does not reduce mucosal carriage of pathogenic serotypes. The worldwide increase in the number of pneumococcal isolates with multiple drug resistance makes prevention of pneumococcal infections important.

1.3.2.2 Conjugate vaccine

Limitations of polysaccharide vaccines have led to the development of protein conjugate vaccines. These act like T-cell-dependent antigens, and they induce an immunological memory. A 7-valent pneumococcal conjugate vaccine was licensed in the United States in 2000, and recommended for routine use in children younger than 2 years of age, an age group who do not respond to polysaccharide vaccines (ACIP 2000). The product is a solution of saccharides of the capsular antigens of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F conjugated to non-toxic diphtheria CRM_{197} protein. These seven pneumococcal serotypes constitute 60-95% of disease-causing strains in children, depending on the population studied (Hattotuwa and Hind 1997), but they represent less than 50% of those found in adults (Jette and Lamothe 1989). In any case there would appear to be cross-protection among related serotypes (Shinefield and Black 2000). Vaccination of both healthy and chronically ill children has been associated with a significant reduction in invasive infections due to *Streptococcus pneumoniae* (Whitney et al. 2006, Black et al. 2007).

Considerably fewer trials have been published on pneumococcal conjugate vaccine in adults. According to two studies made in healthy adults there was no advantage over the polysaccharide vaccine in terms of immunogenicity and there were more local vaccine reactions (Powers et al. 1996, Shelly et al. 1997). In contrast, another study showed the pneumococcal conjugate vaccine to induce a higher antibody response than polysaccharide vaccine, an effect not however seen in human immunodeficiency virus (HIV) - infected persons (Ahmed et al. 1996). Patients with a rare immunodeficient state called ataxia-telangiectasia have low concentrations of pneumococcal antibodies. These patients respond poorly to pneumococcal polysaccharide vaccine, but if first triggered with pneumococcal conjugate vaccine they are able to produce protective antibodies to serotypes included in conjugate vaccine. Conjugate vaccine alone fails to elicit an antibody response in these patients (Stray-Pedersen et al. 2005). In a study with patients previously treated for Hodgkin`s disease, a single dose of 7-valent pneumococcal conjugate vaccine yielded a significantly lower response than 23-valent polysaccharide vaccine (Molrine et

al. 1995). Patients with Hodgkin's disease, who were first primed with pneumococcal conjugate vaccine, evinced significantly better antibody responses after polysaccharide vaccine than those who received only polysaccharide vaccine (Chan et al. 1996). A total of 75% of allogeneic stem cell transplant patients responded to pneumococcal conjugate vaccine, while only 24% responded to polysaccharide vaccine (Pao et al. 2006). In a randomized, double-blind trial in allogeneic stem cell transplant patients, a donor and recipient paired vaccination strategy with pneumococcal conjugate vaccine demonstrated greater immunogenicity than a similar strategy with pneumococcal polysaccharide vaccine (Kumar et al. 2007).

1.3.3 Vaccination studies in CLL

Vaccines in general offer a well-established means of enhancing adaptive immunity, although very little is known of their effectiveness in CLL. From the Medline database starting from 1966, eleven relevant studies (apart from our own) were found concerning vaccinations in CLL (Sinisalo et al. 2003). Their results are summarized in Table 1. The spectrum of tested antigens represented seven different organisms. Most of the studies in question concerned *Streptococcus pneumoniae*, influenza viruses and *Haemophilus influenzae*.

These previous vaccination studies in patients with CLL showed antibody responses to vaccines to be weak (Table 1). Protein vaccines have had some effect (Shaw et al. 1960, Cone and Uhr 1964), but polysaccharides have produced hardly any response (Jacobson et al. 1988, Hartkamp et al. 2001). Normal immunoglobulin concentrations and an early disease stage have slightly improved the response rate (Jacobson et al. 1988, Hartkamp et al. 2001). Mellemgaard and associates (1993) observed no response to pneumococcal polysaccharide vaccine in a relatively large trial involving 40 patients with Binet A stage CLL - this despite the ranitidine adjuvant treatment used. Moderate responses to influenza virus vaccine have been noted, although chemotherapy and hypogammaglobulinemia have reduced the responses (Shaw et al. 1960, Schafer et al. 1979, Gribabis et al. 1994, Neilson et al. 1996, van der Velden et al. 2001), and concomitant ranitidine adjuvant treatment has had no effect on the

outcome (Jurlander et al. 1995). According to one study, a booster vaccination did not markedly improve the result (van der Velden et al. 2001).

With respect to this adjuvant treatment it has been observed that plasma histamine concentrations have been higher in CLL patients than in healthy controls, showing a significant positive correlation to disease duration (Jurlander et al. 1995). In the recent study by van der Velden and colleagues, ranitidine improved vaccination-induced T-cell-dependent antibody responses in CLL patients but had no beneficial effect on the response to vaccination with T-cell independent, polysaccharide antigens (2007a). The effect of ranitidine on the antibody response has been explained by its ability to block the effects of histamine, which, in turn, can inhibit immunoglobulin production and down-regulate lymphocyte proliferation and the production of various cytokines (Jutel et al. 2002).

The immunogenicity of tetanus and diphtheria exotoxins, inactivated mumps virus and killed typhoid bacilli has also been studied in patients with CLL (Shaw et al. 1960, Cone et al. 1964, Mellemgaard et al. 1993, Jurlander et al. 1995). In the case of the other microbes studied, hypogammaglobulinemia has appeared to reduce antibody responses (Shaw et al. 1960).

Studies hitherto have only evaluated seroconversion induced by vaccination, offering no data regarding their efficacy in preventing actual infections or their impact on survival.

Table 1. Vaccination studies in CLL.

Microbe	Vaccine type/ Brand name or manufacturer/ total antigen number	N of responders/ N of patients enrolled	Comments	Reference
Streptococcus pneumoniae	Capsular PS /-/2	1/9	Responder had bacterial pneumonia during study period.	Shaw et al 1960
	Capsular PS /Pneumovax®/23	5/32	Responses were associated with early-stage disease and normal immunoglobulin levels.	Jacobson et al 1988
	Capsular PS /23Pneumovacs®/23	0/40	Ranitidine had no effect on responses.	Mellemgaard et al 1993
	Capsular PS /Pneumovax®/23	5/24	Responses were associated with early-stage disease and normal immunoglobulin levels	Hartkamp et al 2001
	Capsular PS /Pneumovax®/23	3/50	Ranitidine had no effect on responses.	van der Velden et al 2007a
Haemophilus influenzae type b	PS conjugated to tetanus toxoid/ Act-Hib®/1	11/12	Ranitidine enhanced response. Response rate 6/14 in non-ranitidine CLL study group.	Jurlander et al 1995
	PS conjugated to tetanus toxoid/Act-Hib®/1	6/23	Responses were associated with early-stage disease and normal immunoglobulin levels.	Hartkamp et al 2001
	PS conjugated to tetanus toxoid/ Act-Hib®/1	20/50	Ranitidine group response rate 54%, non-ranitidine group 28%.	van der Velden et al 2007a
Influenza (A, B) viruses	Inactivated virus/Parke, Davis and Co./1	9/24		Shaw et al 1960
	Inactivated virus/-/2	8/13	13 patients with lymphoproliferative disease, of whom 8 had CLL, 4 myeloma and 1 hairy cell leukemia. Chemotherapy and low immunoglobulin levels decreased the responses.	Schafer et al 1979
	Inactivated virus/Vaxigrip®/3	35/43	Response was defined as antibody response for at least one antigen. Patients with IgG < 7g/l responded less well.	Gribabis et al 1994
	Inactivated virus/Fluzone/3	9,15*/26	Ranitidine had no effect on responses. *Response rates were 35% for Influenza A and 58 % for Influenza B.	Jurlander et al 1995
	Inactivated virus/Influvac®/3	15/34	Patients with low IgG levels responded poorly.	Neilson et al 1996
	Inactivated virus/Influvac®/3	3,6*/20	*Response rates were 15% for Influenza A and 30% for influenza B after 2 vaccinations. Booster was not beneficial.	van der Velden et al 2001
Clostridium tetanii	Inactivated exotoxin/Tetanus vaccine®/1	15/23	Ranitidine enhanced response. Response rate was 4/17 in non-ranitidine CLL study group.	Mellemgaard et al 1993
	Inactivated exotoxin/ Act-Hib®/1	17/26	Ranitidine had no effect on response.	Jurlander et al 1995
	Inactivated exotoxin/ Act-Hib®/1	18/50	Ranitidine group response rate 68%, non-ranitidine group 24%.	van der Velden et al 2007
Corynebacterium diphtheriae	Inactivated exotoxin / National Drug Co./1	8/24		Shaw et al 1960
	Inactivated exotoxin(KP 59A) /Massachusetts Department of Public Health/1	1/10		Cone et al 1964
Mumps virus	Inactivated virus/ Lederle Laboratories/1	9/24		Shaw et al 1960
Salmonella typhii	Killed bacilli/ Lederle Laboratories/1	12/24		Shaw et al 1960

PS, polysaccharide; Hib, *Haemophilus influenzae* type b

AIMS OF THE STUDY

In this series responses to vaccine antigens in patients with CLL were assessed. Vaccine antigens included tetanus toxoid, unconjugated pneumococcal polysaccharide and conjugated Hib and conjugated pneumococcal antigens.
We also evaluated factors predicting the response.

The specific aims were:

1. to evaluate the antibody response to three types of vaccine antigen: protein, polysaccharide and conjugated protein-polysaccharide antigen in patients with CLL (**I**)

2. to determine the factors affecting antibody response to conjugated polysaccharide vaccine (**II, IV**)

3. to evaluate the effect of IgHV mutation status on humoral immunity parameters and vaccine responses in patients with CLL (**III**)

4. to evaluate the antibody response to pneumococcal conjugate vaccine in patients with CLL (**IV**)

MATERIALS AND METHODS

3.1. Ethical considerations

The study protocols were approved by the ethical committee of Pirkanmaa Hospital District. The work was done according to the Helsinki Declaration. Written informed consent was obtained from all subjects and controls.

3.2. Subjects and controls

3.2.1 Study I

The study population here consisted of a cohort of 31 consecutive patients with CLL (22 males and 9 females), aged 66 (median, range 48-80 years), referred to the hematology outpatient clinic in Tampere University Hospital. The diagnosis and staging of CLL were based on standard clinical, morphological and immunophenotyping criteria (Cheson et al. 1996). All patients had the B-cell phenotype. Disease stage according to Binet`s classification was A in 14, B in 8 and C in 9 patients. Median duration of disease had been 3 years (range 0.5-14 years). At the time of vaccination 7 patients were on chemotherapy, 10 received steroids and 14 had no treatment. Hypogammaglobulinemia (S-IgG <6.7 g/l) was detected in 13 patients, but only 2 had IgG concentrations below 4 g/l. Splenectomy had been performed in one case.

For control purposes, 25 immunologically healthy age and sex-matched persons with no hematological disorders were included in the study.

Histories of infections within a period of two years prior to vaccination were recorded in all cases with CLL. Participants completed a questionnaire on their history of infections and vaccinations and the data were checked and completed by personal interview and examination by a clinical hematologist. Infections were classified as severe if parenteral antimicrobial therapy and hospitalization were needed, and moderate if there were three or more oral antibiotic therapies per year without hospitalization.

To avoid local reactions vaccination was not repeated if any of the investigated vaccines had been given to the study subject within the last five years. Post-vaccination blood samples were not obtained from two patients and one patient was treated with intravenous immunoglobulin before the second blood sample was taken. These three subjects were excluded from the study. Of the patients with CLL, 27 were included in the pneumococcal, 28 in the Hib and 19 in the tetanus-diphtheria part of the study. Of controls, 25 were accepted for the pneumococcal and Hib and 15 for the tetanus-diphtheria part of the study. Demographic and immunological characteristics of patients and controls are shown in Table 2.

3.2.2 Study II

In this investigation we assessed which of the demographic and immunological factors predict a favorable vaccine response and lead to achievement of protective antibody concentrations with Hib conjugate vaccine in 28 CLL patients who participated in study **I**.

Table 2. Demographic and immunological characteristics of patients with CLL and controls (study **I**, **II**). The values are expressed as medians (quartiles) if not otherwise stated.

Parameter (normal values)	**CLL patients** (N=28)	**Controls** (N=25)	**p-value**
Sex, m/f	20/8	15/10	0.402
Age, years	66 (58-64)	59 (55-66)	0.069
Serum IgG, g/l (6.77-15)	8.0 (5.3-9.9)	11.2 (9.5-12.6)	<0.001
Serum IgG1, g/l (5.2-12.7)	5.1 (3.0-6.5)	6.7 (5.7-8.2)	0.003
Serum IgG2, g/l (1.43-5.6)	1.73 (1.12-2.28)	3.37 (2.55-4.11)	<0.001
Serum IgG3, g/l (0.11-0.85)	0.22 (0.12-0.43)	0.25 (0.19-0.40)	0.232
Serum IgG4, g/l (0.030-2.00)	0.17 (0.06-0.40)	0.37 (0.23-0.75)	0.033
Serum IgA, g/l (0.52-4.84)	1.17 (0.36-1.57)	2.12 (1.54-2.70)	<0.001
Serum IgM, g/l (0.36-2.84)	0.31 (0.15-0.70)	1.01 (0.74-1.44)	<0.001
CD4, $x10^9$/l (0.40-1.61)	1.30 (0.65-2.01)	0.77* (0.58-0.93)	0.027
CD8, $x10^9$/l (0.22-1.13)	1.03 (0.62-1.95)	0.69* (0.51-0.83)	0.011
CD4/CD8, ratio (0.8-3.7)	1.00 (0.82-1.75)	1.11* (1.00-1.56)	0.404

*N=22

3.2.3 Study III

Here the IgHV mutation status - unmutated versus hypermutated - was investigated in 33 CLL patients, and its possible correlation to humoral immunity parameters and vaccine responses was analyzed. These patients were from the hematology outpatient clinic of Tampere University Hospital, and 16 of them had participated in studies **I** and **II**. Demographic characteristics of the CLL patients with M-CLL and UM-CLL are shown in Table 3. Disease progression was more advanced among patients with unmutated IgHV genes compared to those with mutated, and they were diagnosed more frequently as Binet stage B or C. UM-CLL patients tended to be more frequently anemic than the M-CLL patients, which may be associated with more advanced disease state.

Table 3. Demographic characteristics of the CLL patients with mutated (M) or unmutated (UM) immunoglobulin heavy chain variable (IgHV) genes (study **III**). The values are expressed as medians and ranges if not otherwise stated.

Parameter (normal values)	**M-CLL** (N=11)	**UM-CLL** (N=22)	**p-value**
Sex, m/f	9/2	16/6	0.687
Age, years	64 (48-73)	68 (53-79)	0.174
Binet, A/B/C	8/1/2	7/9/6	0.067
Binet, A/BC	8/3	7/15	0.031
Binet, AB/C	9/2	16/6	0.454
Progression, slow or intermediate/fast	7/2*	7/10**	0.085
Chemotherapy and/or steroids, yes/no	3/8	6/16	0.667
Infections, severe/moderate/mild or no	1/1/9	4/4/14	0.563
Leukocyte count, $x10^9$/l (3.4-8.2)	95 (56-168)	105 (40-323)	0.499
Lymphocyte count, $x10^9$/l (1.2-3.5)	93 (46-142)	90 (36-310)	0.445
Hemoglobin, g/l (117-167)	130 (108-159)	117 (73-151)	0.126
Anemia (Hb <100), yes/no	0/11	6/14	0.067
Platelet count, $x10^9$/l (150-360)	181 (98-258)	176 (22-453)	0.894
Thrombocytopenia (<100), yes/no	1/10	3/19	0.593
Beta-2-microglobulin, mg/l (0.8-2.8)	3.3 (1.9-5.9)	3.9 (2.0-8.1)	0.252

* N=9, ** N=17

3.2.4 Study IV

This study population comprised a cohort of 52 consecutive patients with CLL (31 males and 21 females), aged 65 years (median, range 43-84 years), who were referred to the hematology outpatient clinics of Tampere or Turku University Hospitals. Clinical and laboratory characteristics of the patients are shown in Table 4. Hypogammaglobulinemia (S-IgG below 6.7 g/l) was detected in 14

patients, but only 1 patient had IgG value <4 g/l. Splenectomy had been performed on one patient.

For control purposes 25 age- and sex-matched immunologically and hematologically healthy persons from the cardiac outpatient clinic of Tampere University Hospital were recruited for the study (median age 66, range 52-77, 16 males, and 9 females). Since post-vaccination samples were unavailable in the case of 3 CLL patients and 1 control, 49 patients with CLL and 24 controls were included in the final study population. None of the patients or controls had previously received a pneumococcal vaccine.

Table 4. Clinical and laboratory characteristics of the CLL patients recruited for study **IV**. The values are expressed as medians and ranges if not otherwise stated.

Character (normal values)	**CLL patients** N=49
Sex, m/f	30/19
Age, years	65 (43-84)
Disease duration, years	2 (0.1-21)
Binet, A/B/C	39/9/1
*Past or ongoing chemotherapy, yes/no	11/38
Lymphocyte count, $x10^9$/l (1.2-3.5)	17.5 (0.9-339.8)
Platelet count, $x10^9$/l (150-360)	176 (70-581)
Hemoglobin, g/l (117-167)	137 (105-169)
Serum total IgG, g/l (6.77–15)	8.40 (3.68-16.69)
Serum IgG1, g/l (5.2–12.7)	6.40 (2.5-13.20)
Serum IgG2, g/l (1.43-5.6)	2.34 (0.69-5.53)
Serum IgG3, g/l (0.11-0.85)	0.34 (0.04-1.23)
Serum IgG4, g/l (0.030-2.00)	0.16 (0.00-1.70)
Serum IgA, g/l (0.52-4.84)	0.40 (0.05-5.81)
Serum IgM, g/l (0.36-2.84)	0.96 (0.25-4.56)

* Only 1 patient was on chemotherapy at the time of vaccination

3.3. Vaccines and sampling schedules

Pneumococcal polysaccharide (Pnu-Immune 23®, Wyeth Pharma, Munster, Germany), Hib conjugate (HibTITER®, Wyeth Manufacturing, England) and Tetanus-diphtheria toxoid vaccine (Tetanus-d-rokote®, National Public Health Institute, Helsinki, Finland), in 0.5 ml doses, were used in study **I**. Pnu-Immune 23® 0.5 ml contains 25µg of pneumococcal polysaccharides types 1 to 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F. HibTITER® contains 10 µg of Hib polysaccharide conjugated to 30 µg of tetanus toxoid as carrier protein, and Tetanus- d-rokote® contains 2 IU diphtheria toxoid and 20 IU tetanus toxoid. Individual vaccines were given intramuscularly at separate sites on the same visit.

In study **IV**, 7–valent pneumococcal conjugate vaccine (Prevenar®, Wyeth Lederle Vaccines), which contains capsular polysaccharides of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F individually conjugated to diphtheria CRM_{197} protein were used. The product is manufactured as a liquid preparation. Each 0.5 ml dose contains 2 µg of polysaccharides type 4, 9V, 14, 18C, 19F, and 23F, 4 µg of polysaccharides type 6B (16 µg total polysaccharide), approximately 20 µg of CRM_{197} carrier protein, and 0.125 mg of aluminum as phosphate adjuvant. Patients and controls received one subcutaneous deltoid injection of Prevenar®. Venous blood samples were taken before and four weeks after the vaccination in studies **I** and **IV**. Serum was separated by centrifugation and stored at -20°C.

3.4. Methods

3.4.1 Determination of antibodies

In study **I**, IgG-antibodies to pneumococcal serotypes 1, 3, 6B, 14, 19F and 23F, and to *Haemophilus influenzae* were determined by enzyme immunoassay (EIA)

(Käyhty et al. 1995, Kurikka et al. 1995). The total anti-pneumococcal polysaccharide antibody concentration was calculated as a sum of serotype-specific antibody concentrations. For the determination of IgG antibodies to tetanus toxoid, a slightly modified double antigen EIA was used (Kristiansen et al. 1997). Since diphtheria toxoid worked as a carrier in the Hib conjugate preparation, the specific antibody concentrations against it were not evaluated.

In study **IV**, serum concentrations of IgG antibody to pneumococcal polysaccharides 4, 6B, 9V, 14, 18C, 19F and 23F were measured by EIA (Käyhty et al. 1995) with a 22F capsular polysaccharides adsorption step (Concepcion and Frasch 2001).

Results are given as µg/ml calculated on the basis of the assigned IgG values of the 89-SF reference serum (Quataert et al. 1995). The detection limits were: types 4 and 18C; 0.05, types 6B and 9V; 0.06, type 23F; 0.07, type 14; 0.12, and type 19F; 0.13 µg/ml, respectively.

3.4.2 IgHV mutation analysis

In study **III**, IgHV gene polymerase chain reaction (PCR) amplification followed by nucleotide sequencing was performed using six family-specific IgHV primers and one JH primer as described elsewhere (Vilpo et al. 2003). To distinguish monoclonal PCR products from polyclonal, single-strand conformation polymorphism analysis was performed using Gene Phor system electrophoresis (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Clonal PCR products from 38 rearrangements were sequenced directly using Big Dye Terminator Cycle Sequencing Reaction Kits (Perkin-Elmer, ABI, Foster City, CA, USA). All sequence reactions were analyzed using an automated DNA sequencer (ABI 377, Applied Biosystems, Foster City, CA). IgHV gene sequences deviating more than 2% from the closest aligned germ line gene published in the Gen Bank, V-BASE or IMGT databases were defined as mutated, whereas sequences displaying 2% or less mutations were considered unmutated.

3.4.3 Determination of immunological parameters

In studies **I**, **II** and **III**, serum concentrations of IgG, IgM, and IgA and IgG subclasses were determined using a Behring Nephelometer (Behringwerke AG, Marburg, Germany) according to manufacturer's instructions. The serum concentration of MBL was measured using an in-house EIA, as described elsewhere (Aittoniemi et al. 1996). The plasma beta-2-microglobulin concentration was determined by a Cobas Core analyser using an enzyme immunoassay technique (Cobas Core beta-2-microglobulin EIA, Hoffmann-La Roche, Switzerland). The anti-A and anti-B titres were determined applying a standard tube technique for ABO grouping, as previously described (Vengelen-Tyler 1996).

3.4.4 Statistical analyses

In study **I**, pre-vaccination antibody concentrations and proportionate changes between groups were compared by Mann-Whitney U or Kruskall-Wallis test, and significance in antibody responses was evaluated by Wilcoxon's matched pairs test. Proportionate changes had been calculated by dividing each patient's post-vaccination antibody concentration by that pre-vaccination. The results are expressed as medians and quartiles.

In studies **II** and **III**, proportions were compared by Fisher's exact or chi-square, and median concentrations by Mann-Whitney U-test. Logistic regression analysis was applied to identify the independent effect of each parameter when appropriate.

In study **IV**, antibody concentrations and proportionate changes between groups were compared by independent-samples t-test and the significance of responses within groups by paired-samples t-test. For statistical analyses, data distributions were normalized by log10-transformation. However, the values in the tables are expressed as true geometric mean concentrations (GMC) and 95% confidence intervals (CI) calculated through these transformations. Proportions deduced from antibody concentrations were compared by Pearson's chi-square test.

RESULTS

4.1. Responses to different types of antigens (study I)

In this study, antibody responses to tetanus toxoid, unconjugated pneumococcal polysaccharide vaccine and Hib conjugated vaccine antigens were assessed.

Pre-vaccination antibody concentrations and proportionate changes against different antigens in patients with CLL and controls are shown in Table 5. The pre-vaccination antibody concentrations against Hib polysaccharide antigen were lower in patients with CLL than in controls ($p=0.022$), but there were no differences in pre-vaccination pneumococcal polysaccharide antibody concentrations ($p=0.912$). The only significant antibody response in the CLL patients was that observed to Hib polysaccharide antigen: six (21%) out of 28 patients were able to develop an antibody concentration considered suggestive of protection (≥1 µg/ml) (Käyhty et al. 1983), and the post-vaccination antibodies were above this concentration in 54% of patients. In control subjects all responses were significant and more intense than those observed in patients with CLL.

Individual antibody responses against different types of antigens in patients with CLL are illustrated in Figure 3. When comparing the CLL patient subgroups with respect to Binet class, total serum IgG and pre-vaccination antibody concentrations, the proportionate antibody response to tetanus toxoid antigen was somewhat higher in those with Binet class A ($p=0.031$) and in those with normal serum IgG concentrations ($p=0.021$). A weak, albeit statistically significant difference (higher proportionate change) was also detected against pneumococcal polysaccharide antigen in patients with a high pre-vaccination antibody concentration (>median) compared to those with a low concentration ($p=0.042$).

Among patients with CLL, severe or moderate infections were recorded in 13 (46%). Infections were classified as severe in five patients and moderate in eight. No statistically significant difference in vaccination response was detected in CLL patients with or without infections.

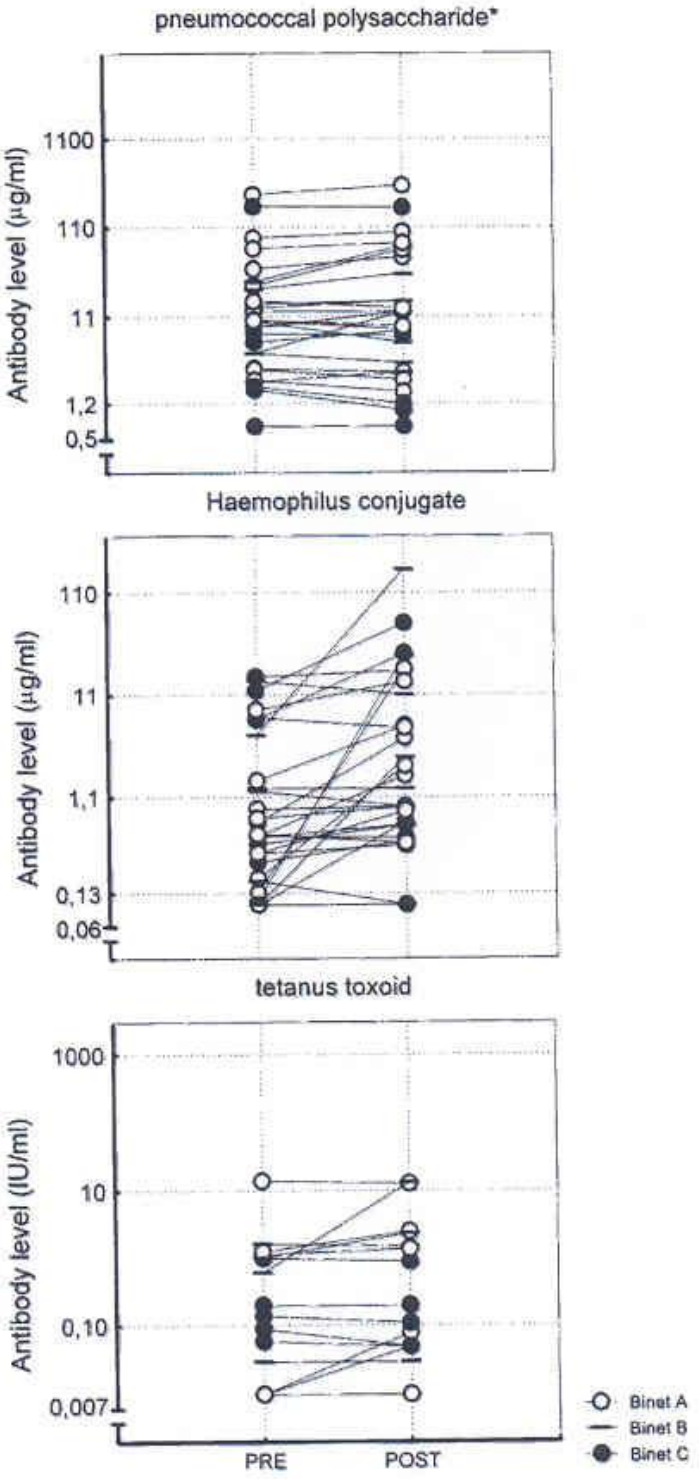
Table 5. Medians and quartiles of antibody concentrations before vaccination, and proportionate changes in patients with CLL and controls in study **I**.

Vaccine antigen	**Pre-vacc. concentration median (quartiles)**		**p-value[a]**	**Proportionate change median (quartiles)**		**p-value[a]**
	CLL	**Controls**		**CLL**	**Controls**	
Pneumococcal polysaccharide (µg/ml)	10.1 (2.92-24.6)	9.41 (6.03-15.0)	0.912	1.00 (0.80-1.35)	4.68 (3.25-7.70)**	<0.001
Serotype 1	0.77 (0.28-3.42)	1.13 (0.42-1.41)	0.956	1.00 (0.84-1.36)	3.81 (2.93-5.52)**	<0.001
Serotype 3	0.63 (0.27-2.39)	1.14 (0.63-1.42)	0.533	0.93 (0.79-1.19)	4.34 (2.49-7.93)**	<0.001
Serotype 6B	1.02 (0.33-2.64)	1.40 (0.68-3.06)	0.521	0.99 (0.75-1.88)	2.41 (1.30-4.25)*	<0.001
Serotype 14	1.46 (0.45-2.87)	0.53 (0.31-1.27)	0.094	1.00 (0.83-1.29)	6.82 (1.37-21.6)**	<0.001
Serotype 19F	2.40 (0.77-8.30)	2.31 (1.39-3.81)	0.784	1.06 (0.73-1.40)	3.61 (2.46-10.1)**	<0.001
Serotype 23F	1.56 (0.33-3.91)	1.70 (0.73-2.91)	0.898	0.90 (0.75-1.12)	3.60 (1.54-7.27)**	<0.001
Haemophilus influenzae conjugate (µg/ml)	0.48 (0.22-3.06)	1.54 (1.02-3.71)	0.022	1.58 (0.99-5.47)*	42.1 (11.9-105)**	<0.001
Tetanus toxoid (IU/ml)	0.12 (0.01-1.03)	0.19 (0.01-3.61)	0.547	1.00 (0.88-2.00)	3.33 (1.39-72.1)*	0.005

The significance of proportionate increase between pre- and post-vaccination samples, Wilcoxon's matched pairs test, *P <0.05; **P <0.001

[a]Mann-Whitney U-test

Figure 3. Antibody responses to different types of antigens in patients with CLL (study **I)**. * Expressed as sum of serotype-specific antibody levels.



4.2. Factors affecting response (study II)

In study II factors predictive of antibody response to conjugated Hib vaccine were assessed.

Many of the CLL patients had compromised humoral immunity as measured by serum concentrations of immunoglobulins (Table 2). Cell-mediated immunity was fairly normal according to T-lymphocyte counts. Although the absolute count of CD4 and CD8 lymphocytes was higher in patients with CLL than in controls, no difference in CD4/CD8 ratio was observed. Results of logistic regression analysis identifying the independent effect of demographic and immunological findings on Hib antibody concentrations and responses are shown in Table 6. Lower age was the most significant predictor with respect to protective anti-Hib antibody concentrations. High IgG1 and IgA concentrations were also associated with this effect.

A high IgM concentration best predicted significant vaccination response. The disease stage according to Binet`s classification or hypogammaglobulinemia was not a significant predictor for response in this study.

Table 6. Results of logistic regression analysis identifying the independent effect of demographic and immunological findings on *Haemophilus influenzae* type b (Hib) antibody concentrations and response to vaccination (study **II**). The results are expressed as medians (quartiles) if not otherwise stated.

Parameter (normal values)	**Concentration of ≥1 µg/ml or significant response (2-fold increase)**		**p-value**
	Yes	No	
Pre-vaccination antibody concentration >1 µg/ml	**N= 10**	**N=18**	
Age, years	59 (55-66)	68 (63-71)	0.017
Serum IgG1, g/l (5.2-12.7)	6.1 (3.4-8.1)	4.5 (2.7-5.6)	0.048
Post-vaccination concentration >1 µg/ml	**N=15**	**N=13**	
Age, years	61 (55-67)	68 (66-70)	0.053
Serum IgA, g/l (0.52-4.84)	1.29 (0.81-1.97)	0.89 (0.15-1.30)	0.062
Absolute increase >1 µg/ml	**N=12**	**N=16**	
Serum IgM, g/l (0.36-2.84)	0.55 (0.22-0.78)	0.27 (0.13-0.34)	0.041
CD8, $x10^9$/l (0.22-1.13)	0.99 (0.54-2.34)	1.14 (0.62-1.86)	0.064
Age, years	60 (56-67)	68 (64-71)	0.115
Proportionate increase >2x	**N=12**	**N=16**	
Serum IgM, g/l	0.55 (0.25-0.81)	0.25 (0.13-0.34)	0.052
Age, years	59 (56-67)	68 (64-71)	0.054

4.3. Effect of IgHV mutation status (study III)

The effect of IgHV mutation status on vaccine response and other humoral immunity parameters was evaluated in this study.

Immunologic characteristics of the CLL patients with mutated or unmutated IgHV gene are shown in Table 7. No differences were detected in immunoglobulin isotype or MBL concentrations. Thus, no signs of increased humoral immunodeficiency could be detected among UM-CLL patients

compared to M-CLL. Nor were any differences found in the incidence of severe infections in these two groups; the population studied was however relatively small.

In order to evaluate the current state of humoral immune function, we investigated responses to Hib conjugate vaccine and measured anti-ABO blood group IgM antibodies in CLL patients with different mutation status. However, no significant differences in Hib vaccination responses or anti-ABO IgM antibody concentrations were indicated between UM-CLL and M-CLL. Positive vaccine responses were seen in both groups, although the patients in the unmutated group belonged more to Binet B and C classes.

Table 7. Immunologic characteristics of the CLL patients with mutated (M) or unmutated (UM) immunoglobulin heavy-chain variable (IgHV) genes (study **III**). Concentrations are expressed as medians (quartiles) if not otherwise stated.

Parameter (normal values)	**M- CLL (**N=11)	**UM- CLL** (N=22)	**p-value**
Serum IgG, g/l (6.77-15.00)	8.5 (6.8-13)	8.0 (3.1-15)	0.229
Serum IgG1, g/l (5.2-12.7)	6.0 (3.7-10)	5.4 (2.6-10)	0.349
Serum IgG2, g/l (1.43-5.6)	2.2 (0.63-6.5)	1.9 (0.33-3.7)	0.260
Serum IgG3, g/l (0.11-0.85)	0.28 (0.14-0.53)	0.36 (0.05-1.0)	0.541
Serum IgG4, g/l (0.030-2.00)	0.24 (0.041-1.5)	0.17 (0.005-1.5)	0.647
Serum IgM, g/l (0.36-2.84)	0.27 (0.09-0.78)	0.37 (0.09-8.3)	0.285
Serum IgA, g/l (0.52-4.84)	1.5 (0.31-3.1)	1.0 (0.27-4.5)	0.340
MBL, mg/l * N=32	2.9 (0.69-11)	4.6 (0.63-14)	0.275
Anti-ABO antibodies			
ABO blood group, A/B/AB/O	4/1/1/3	7/2/5/7	0.865
Anti-A antibody titer, N=12	8 (4-8)	6 (2-16)	0.656
Anti-B antibody titer, N=19	4 (0-16)	3 (0-16)	0.193
Antibody response against Hib ** **vaccination, N=16**			
Pre-Hib, µg/ml	0.70 (0.10-12.2)	0.49 (0.10-15.2)	0.958
Pre-Hib >1 µg/ml, yes/no	3/6	3/4	0.549
Post-Hib, µg/ml	1.35 (0.60-55.3)	2.75 (0.40-186)	0.710
Post-Hib >1 µg/ml, yes/no	5/4	4/3	0.671
Absolute increase, µg/ml	0.21 (-1.37-43.1)	-0.02 (-4.12-182)	0.791
Absolute increase >1 µg/ml, yes/no	3/6	3/4	0.549
Relative increase, ratio	1.54 (0.79-12.5)	0.96 (0.70-244)	0.791
Relative increase >2-fold, yes/no	4/5	3/4	0.671

* Mean 4.48 and median 4.02 mg/l in Finnish adults (Aittoniemi et al. 1996)

** *Haemophilus influenzae* serotype b

4.4. Pneumococcal conjugate vaccine (study IV)

Antibody responses to pneumococcal conjugate vaccine were evaluated in this study.

Pre- and post-vaccination concentrations of antibody against pneumococcal polysaccharide antigens are shown in Table 8. No difference in pre-vaccination antibody concentrations was observed between CLL patients and controls. Among the patients with CLL, advanced stage of disease (Binet B or C), past or ongoing chemotherapy, use of corticosteroids or history of increased susceptibility to infections were not associated with decreased pre-vaccination antibody concentrations. However, concentrations of antibody against certain serotypes (9V and 18C) were lower in CLL patients with hypogammaglobulinemia compared to those with normal IgG concentration. After vaccination, the antibody concentrations were significantly lower in CLL patients than in controls for all serotypes.

Response rates and the proportions of pre- and post-vaccination antibody concentrations suggestive of protection against pneumococcal polysaccharide antigens of the conjugate vaccine in patients with CLL and controls are shown in Table 9. An antibody concentration of at least 0.35µg/ml was held suggestive of protection, since this value is used at the WHO pneumococcal reference laboratory as a protective concentration, and is based on a pooled analysis of published studies (WHO 2005). A significant increase in antibody concentrations was detected in both groups for all antigens after vaccination. However, the rise was significantly higher among the controls, varying from 8.3- to 23-fold depending on the antigen, compared to a 1.7- to 3.5-fold change in patients with CLL. A significant response, defined as an at least 2-fold increase and a post-vaccination concentration of at least 0.35µg/ml, was observed in 20-47% of CLL patients depending on serotype. In controls the percentage varied from 75% to 88%. In patients with CLL, the proportions of antibody concentrations suggestive of protection (≥0.35 µg/ml) rose from 14-84% to 49-92% after vaccination depending on serotype, while in controls the corresponding percentages were 8-92% and 79-100%, respectively. The use of corticosteroids

or a history of infections had no effect on response frequencies in patients with CLL. However, significant response rates were lowered in the case of certain serotypes in the subgroups of patients with advanced disease (Binet class B/C; serotypes 4, 9V), past or ongoing chemotherapy (serotypes 4, 6B, 9V, 18C) and hypogammaglobulinemia (serotypes 9V, 18C, 19F).

The distributions of the numbers of significant responses in individual patients with CLL and controls are shown in Figure 4. Among the controls, a significant response to at least 6 antigens was achieved in 71% of cases (17/24), while in the CLL patients the corresponding percentage was 24% (12/49). However, if the vaccine had been administered at an early stage in the disease (Binet A), before commencement of chemotherapy and the development of hypogammaglobulinemia, 39% (11/28) of these CLL patients developed a significant response to at least 6 antigens, as against 5% (1/21) in other patients.

Figure 4. Distribution of the number of significant responses (proportionate increase at least 2-fold and post-vaccination concentrations at least 0.35µg/ml) against pneumococcal vaccine serotypes in individual patients with CLL and controls (study **IV**).

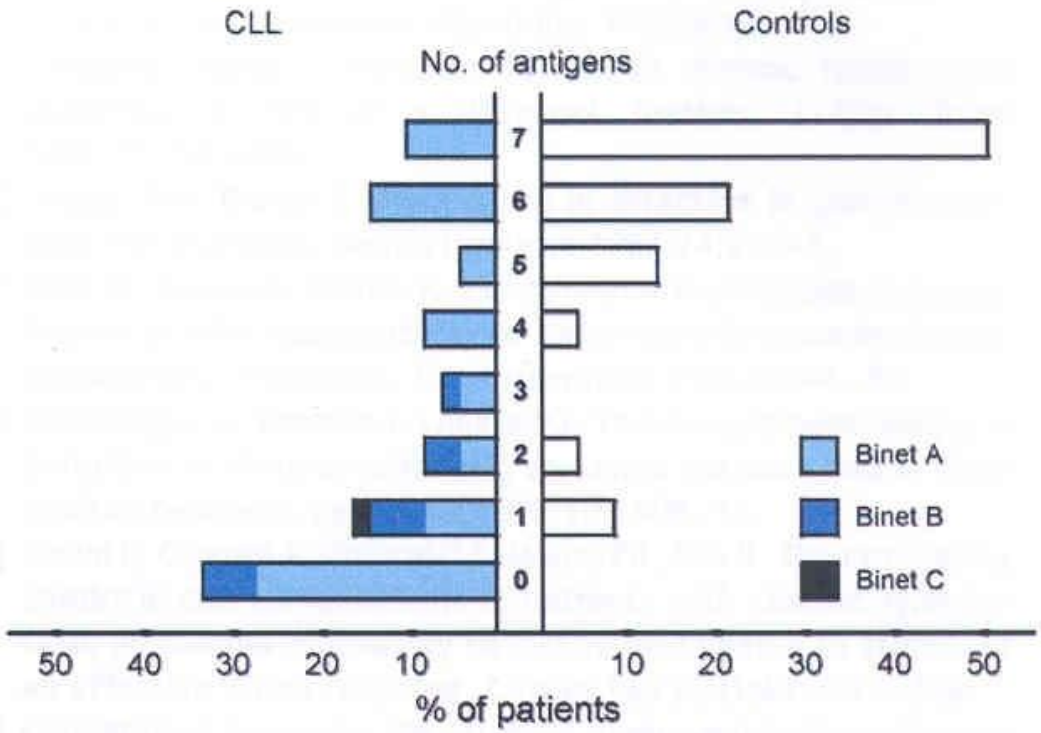


Table 8. Pre- and post-vaccination concentrations of antibody to pneumococcal polysaccharide antigens of 7-valent conjugate vaccine in CLL patients and controls. The concentrations are expressed as geometric means (GMC) with 95% confidence intervals (CI) prior to and one month after vaccination (study **IV**).

Serotype	**Pre-vaccination antibody concentration GMC, µg/ml (95% CI)**		***P-value***[a]	**Post-vaccination antibody concentration GMC µg/ml (95% CI)**		***P-value***[a]
	CLL (N=49)	Controls (N=24)		CLL (N=49)	Controls (N=24)	
4	0.13 (0.09-0.18)	0.11 (0.08-0.15)	0.535	0.32 (0.19-0.54)	1.64 (0.82-3.28)	<0.001
6B	0.30 (0.21-0.44)	0.35 (0.19-0.63)	0.704	0.63 (0.36-1.11)	4.44 (1.75- 11.27)	<0.001
9V	0.37 (0.26-0.53)	0.33 (0.19-0.57)	0.738	1.12 (0.68-1.86)	7.63 (3.24-17.96)	<0.001
14	0.67 (0.47-0.94)	0.77 (0.44-1.33)	0.655	1.12 (0.69-1.82)	6.34 (2.96-13.58)	<0.001
18C	0.88 (0.61-1.27)	1.31 (0.75-2.27)	0.232	2.42 (1.49-3.92)	15.73 (8.14-30.41)	<0.001
19F	1.10 (0.77-1.56)	0.70 (0.44-1.11)	0.149	2.41 (1.48-3.94)	7.66 (3.23-18.18)	<0.001
23F	0.58 (0.39-0.86)	0.71 (0.40-1.28)	0.558	2.02 (1.14-3.56)	9.29 (4.08-21.13)	0.001

[a] independent-samples t-test

Table 9. Response rates[a], and proportions of pre- and post-vaccination antibody concentrations suggestive of protection (≥0.35 µg/ml[c]) against pneumococcal polysaccharide antigens of 7-valent pneumococcal conjugate vaccine in CLL patients and controls (study **IV**).

Serotype	**Response rate[a] (N)**		***P-value[b]***	**Pre-vacc. concentration >0.35 µg/ml[c] (N)**		***P-value[b]***	**Post-vacc. concentration >0.35 µg/ml[c] (N)**		***P-value[b]***
	CLL (N=49)	Controls (N=24)		CLL (N=49)	Controls (N=24)		CLL (N=49)	Controls (N=24)	
4	17 (35%)	20 (83%)	<0.001	7 (14%)	2 (8%)	0.467	24 (49%)	19 (79%)	0.014
6B	17 (35%)	18 (75%)	0.001	21 (43%)	13 (54%)	0.363	28 (57%)	20 (83%)	0.027
9V	21 (43%)	21 (88%)	<0.001	28 (57%)	11 (46%)	0.363	37 (76%)	22 (92%)	0.100
14	10 (20%)	18 (75%)	<0.001	34 (69%)	18 (75%)	0.619	35 (71%)	21 (88%)	0.127
18C	19 (39%)	20 (83%)	<0.001	40 (82%)	22 (92%)	0.260	43 (88%)	24 (100%)	0.074
19F	21 (43%)	21 (88%)	<0.001	41 (84%)	18 (75%)	0.377	45 (92%)	23 (96%)	0.525
23F	23 (47%)	19 (79%)	0.009	27 (55%)	17 (71%)	0.197	38 (78%)	22 (92%)	0.139

[a]proportionate increase at least 2-fold and post-vaccination concentration at least 0.35 µg/ml; [b]chi-square test; [c]WHO-recommended protective concentration for anticapsular antibodies to the 7 serotypes in Prevenar applicable for assessing efficacy of pneumococcal conjugate vaccines against invasive pneumococcal disease in infants

DISCUSSION

5.1 Background to the study

Very few studies have been undertaken concerning microbial vaccinations in patients with hematological malignancies - except for patients with stem-cell transplantations (Parkkali et al. 1996, Patel et al. 2007, van der Velden et al. 2007b). There has been no consensus on vaccination in CLL patients, and clinical practice has varied greatly between institutions. Vaccination of patients may have led to a false sense of protection in uncertain situations.

5.2 Vaccine-induced antibodies

In our study **I** antibody responses against different vaccination antigens were much weaker in patients with CLL compared to those observed in controls. This finding is in accord with those in earlier studies concerning the efficacy of pneumococcal polysaccharide and protein (influenza and toxoid) vaccines in patients with CLL (Larson and Tomlinson 1953, Shaw et al. 1960, Jacobson et al. 1988, Gribabis et al. 1994). Here, the most significant response in CLL patients was detected against Hib polysaccharide antigen conjugated to protein-carrier. Despite the relatively small number of patients in the tetanus toxoid vaccination group, low reactivity to this antigen could also be demonstrated in certain subgroups. Plain polysaccharides as T-cell independent antigens seemed to be ineffective in patients with CLL.

Although conjugated pneumococcal vaccines have proved to be highly immunogenic in patients with autologous and allogeneic stem cell transplantations (Meisel et al. 2007, van der Velden et al. 2007b), their efficacy

has not previously been studied in patients with CLL. In study **IV**, the antibody responses induced by one dose of pneumococcal conjugated vaccine were superior compared to those with pneumococcal polysaccharide vaccine in study **I**. Nonetheless, the responses to Hib and pneumococcal conjugate antigens were clearly lower in CLL patients than in controls. On the other hand, a relatively high proportion of patients reached concentrations considered protective after a single dose of conjugate vaccine. The situation may be even better than antibody determinations alone would indicate; in contrast to plain polysaccharide vaccines the conjugate vaccines are able to induce immunological memory, and protection has also been attributed to this circumstance (Goldblatt 2000). These results clearly demonstrate that significant responses to most of the tested antigens may be obtained using novel conjugate vaccines.

It seems logical that CLL patients should evince better response with conjugate than polysaccharide vaccines by reason of the different mechanisms of their actions. Plain polysaccharide vaccines act only via B-cells, while conjugate vaccines also utilize T-cells in their response. Conjugation of a polysaccharide to a protein carrier leads to activation and clonal expansion of carrier-specific T-cells, as well as polysaccharide-specific B-cells (Stein 1992). In our study there were no differences in the amounts or distributions of different T-cell types between CLL patients and control subjects. In fact, CLL patients had higher counts of CD4 and CD8 cells than controls. This might be due to the high proportion of early-stage patients, and the small number of patients with a history of heavy treatment.

In this study we demonstrated - contrary to earlier observations (Chapel and Bunch 1987) - that pre-vaccination pneumococcal antibody concentrations were very similar in CLL patients and controls. Because the risk of pneumococcal infections is higher in CLL patients than in non-CLL subjects, this suggests that pre-vaccination specific antibodies may not be functionally fully active and protective. Immunological defects and poor humoral response probably precede the diagnosis of CLL by several years, in the manner of the premalignant state known as monoclonal B-cell lymphocytosis (Marti et al. 2005). Pneumococcal antibodies induced by polysaccharide and conjugate vaccines may have different concentrations and qualities as evaluated by avidity

or opsonic functional activity (Anttila et al. 1998, Goldblatt et al. 2006). The half-life of vaccine-induced antibodies in CLL patients has not been studied. Immunity to Hib can persist for at least 8 years in children who have received Hib conjugate vaccine, and the persistence depends on repeated antigenic stimuli (Mäkelä et al. 2003). In a recent study by Amanna and associates (2007), the half-lives of antiviral antibodies in healthy persons were notably long: 50 years or more after live viral infections and 11 to 19 years after vaccinations. In persons with immune deficiencies and chemotherapies these antibodies presumably disappear more rapidly.

5.3 The factors affecting antibody response

After Hib conjugate vaccine, 21% of CLL patients developed a significant response. Responses to conjugate antigens were also detected in patients with increased susceptibility to infection. No difference in response with respect to general infection history was detected in the case of Hib conjugate vaccine. This finding suggests that the conjugate vaccines may be effective even in CLL patients with apparent susceptibility to infections. Lower age was the most significant predictor with respect to protective anti-Hib antibody concentration. High IgG1 and IgA concentrations likewise predicted protective efficacy. A high serum polyclonal IgM best predicted significant vaccination response to Hib conjugate vaccine. It may be speculated that the vaccination response to Hib conjugate vaccine resembles the primary humoral response, where IgM predominates (Goldsby et al. 2000) or that the patient's IgM concentration is otherwise a good marker for immunocompetence in CLL.

Neither disease stage according to Binet`s classification nor hypogammaglobulinemia was a significant factor for response to Hib conjugate vaccine. These results diverge from those reported in a study in which patients responding to Hib conjugate vaccine had more often less advanced disease state, normal immunoglobulins and higher total IgG and IgG2 and IgG4 levels than the non-responder group (Hartkamp et al. 2001). Likewise in our study with

pneumococcal conjugate vaccine, the response rates were compromised by advanced disease state, chemotherapy and hypogammaglobulinemia, the rates varying from 20-47%, and only 25% of CLL patients mounted a significant response to at least six antigens. However, almost 40% of patients developed a significant response to at least six out of the seven antigens included in the vaccine if the vaccine had been administered at an early stage in the disease, before commencement of chemotherapy and the development of hypogammaglobulinemia.

The mutation status of the IgHV gene, which is one of the most important prognostic factors in CLL, had no appreciable effect on humoral immunity or vaccine responses in our study with Hib vaccine. Nor did we find any difference in the incidence of severe infections in these two mutational status groups, but our cohort (study **III**) was relatively small. This indicates that the poorer outcome in UM-CLL may be caused by faster progression of the disease and not by defects in immunity and proneness to infections. A group under Delgado in a study involving 280 CLL patients has recently demonstrated that unmutated IgHV gene status was one of the factors associated with a significantly shorter time to first infection, and also to infection-related mortality in CLL patients (Francis et al. 2006).

5.4 Vaccination strategies

The booster effect with repeated vaccine doses has been important at least in children receiving conjugate vaccines, but it has not been studied in CLL. The memory cells of CLL patients may not be functionally normal, as CLL presumably originates from a memory B-cell (Klein et al. 2001). In pediatric patients with allogeneic stem cell transplantation, response rates after two doses of 7-valent pneumococcal conjugate vaccine have been 56% and after three doses almost 75% (Meisel et al. 2007). Stray-Pedersen and associates noted that adult patients with ataxia-telangiectasia failed to respond to a single dose of pneumococcal conjugate vaccine, but if first triggered with 7-valent pneumococcal conjugate vaccine and given 23-valent polysaccharide vaccine

thereafter, they reached relatively good responses for the serotypes included in the conjugate vaccine (Stray-Pedersen et al. 2005). A group in Manchester has studied the effects of two doses of 7-valent pneumococcal conjugate vaccine followed by a dose of the 23-valent polysaccharide vaccine in patients with myeloma (n=24) and in CLL (n= 6). Patients who had received the 23-valent vaccine in the preceding 5 years received only two doses of conjugate vaccine. The investigators found that this regimen was immunogenic only in individuals naive to the polysaccharide vaccine, and adequate concentrations were achieved in 45% of patients, but not until they had received all three vaccinations. After the first dose of conjugate vaccine fewer than 10% responded. No additional response was observed in patients who had previously received polysaccharide vaccine (Greenfield et al. 2007). On the other hand, studies of group A and C meningococcal polysaccharide vaccine have shown that a state of immune tolerance, or hyporesponsiveness, can develop to repeated polysaccharide vaccine antigen exposures (Gold et al. 1975). This phenomenon of hyporesponsiveness could also be a concern for pneumococcal antigens, as recently reviewed (O´Brien et al. 2007).

5.5 Limitations of the study and future directions

There were at least three limitations to this study. First, we used only one dose of conjugated vaccines, so the booster effect and immunological memory were not studied. The trigger and booster effect of conjugated vaccine would warrant investigation in the future. Optimal dosing and strategies combining polysaccharide and conjugated vaccines, and the use of novel adjuvants (Jurlander et al. 1995, Chan et al. 1996, van der Velden et al. 2007a), might further improve the immunogenicity of the vaccines in CLL patients.

Secondly, the functional activities and half-lives of these vaccine-induced antibodies in CLL are unclear. The etiology and function of pneumococcal antibodies in CLL patients should be further studied for example by determining their opsonophagocytic activity. The duration of humoral immunity as measured

by specific antibody concentrations and the avidity of antibodies several months or years after vaccination would be an interesting subject of research.

Thirdly, we studied only the immune response, not the clinical effect of vaccination. The size of our study does not allow assessment of how these vaccine-induced antibodies correlate to infections in clinical practice.

Many other questions remain open. There are no data on pneumococcal serotypes causing infections in patients with CLL. There are a great variety of disease-causing serotypes in different patient populations around the world. Most studies with pneumococcal conjugate vaccine have been carried out in children. In the United States 82% of invasive pneumococcal infections in patients younger than 2 years have been found to be caused by serotypes included in the 7-valent conjugate vaccine, but in patients aged 65 years or older only 56% of cases were due to these serotypes (Robinson et al. 2001). The serotype coverage of the 23-valent polysaccharide vaccine against invasive pneumococcal infections in the study in question was 86% in patients aged 65 years or older. Thus, pneumococcal serotypes among elderly adults are different, but a recent study has shown that pediatric pneumococcal serotypes increase in elderly adults (Feikin et al. 2005). After the introduction of the conjugate vaccine for infants in the United States in 2000, the rate of antibiotic-resistant invasive pneumococcal infections has also decreased in adults who have not received conjugate vaccine, a finding suggesting that the vaccine interrupts the transmission of resistant strains from children to adults (Kyaw et al. 2006). On the other hand, an increase in infections caused by non-vaccine serotypes, especially type 19A, has been a source of concern, but the magnitude of this effect has fortunately been relatively small (Kyaw et al. 2006).

There is clearly call for the development of conjugate vaccines with higher serotype coverage, including the significant serotypes in the elderly population, and evaluation of different protein antigens common to all pneumococcal serotypes as potential vaccine candidates.

The stereotyped B-cell receptors found in UM-CLL patients indicate that selected antigens could play a role in the progression of the clonal expansion and in the clinical course of CLL (Tobin et al. 2004, Tschumper et al. 2008). Which antigens are involved, is unclear, but one interesting preliminary observation is

that CLL cells express antibodies reactive with antigenic epitopes on the surface of common bacteria. Thus bacterial infections could have some role even in CLL progression by intermittently stimulating CLL precursors and potentially clonal evolution (Hatzi et al. 2006).

Purine analogues and monoclonal antibodies are nowadays used even as a first-line therapy in the treatment of poor-prospect CLL patients. This might further reduce antibody responses in CLL patients. For this reason, vaccination at an early stage would be beneficial in these cases, but to prove actual benefit, large clinical vaccination trials are needed. New treatments also induce long-lasting T-cell depletion, which has altered the infection spectrum from bacterial to viral. Herpes and hepatitis viruses as well as influenza viruses are common causative agents of infections among CLL patients. Clearly studies on vaccinations against these types of viral infections are also warranted.

SUMMARY AND CONCLUSIONS

CLL is a disease in which clonal, incompetent B-lymphocytes accumulate in blood, bone marrow and lymph nodes. Due to immunodeficiency patients with CLL are prone to infections, *Streptococcus pneumoniae* being the most conspicuous single pathogen. Very few effective tools are to hand to prevent infections in this patient group. Here we investigated the efficacy of polysaccharide and protein-conjugated polysaccharide vaccine antigens in patients with CLL by measuring antibody responses, and evaluated factors predicting the response.

Plain polysaccharide 23-valent pneumococcal vaccine proved to be practically ineffective in antibody production among patients with CLL. In contrast, conjugated 7-valent pneumococcal-and Hib vaccines seemed to be immunogenic in these patients. Significant responses to vaccine antigens were seen in 20-25% of CLL patients after a single dose of these conjugate vaccines. Factors best predicting significant vaccination response to Hib conjugate vaccine and/or protective antibody concentrations were a high serum IgM concentration and lower age. Mutational status, which divides CLL into two different prognostic groups, did not correlate with response rates to Hib conjugate vaccine.

A significant vaccination response to one dose of pneumococcal conjugate vaccine was obtained in almost 40% of CLL patients if the vaccine had been administered at an early stage in the disease, before initiation of chemotherapy and the development of hypogammaglobulinemia.

Vaccination of CLL patients with pneumococcal or other conjugate vaccines at the time of diagnosis may be warranted, especially since the conjugate vaccine can induce long-lasting, T-cell-dependent immunity against the pathogen. It might also be reasonable to vaccinate patients with poor-

prognosis mutational status independently of disease stage, in the early course of the disease, with conjugate vaccines. Further studies concerning the functional efficiency of antibodies, optimal vaccination schemes, antigens and serotypes used, optimal doses, orders, boosters and the clinical effectiveness and possible disadvantages of vaccination are called for in patients with CLL. Very large clinical trials are clearly needed to establish the real clinical effect on infection-induced mortality in CLL.

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SHORT REPORT

Response to vaccination against different types of antigens in patients with chronic lymphocytic leukaemia

MARJATTA SINISALO,[1] JANNE AITTONIEMI,[2] PETRI OIVANEN,[1] HELENA KÄYHTY,[3] ROSE-MARIE ÖLANDER[3] AND JUHANI VILPO[4] [1]*Department of Internal Medicine and* [2]*Department of Clinical Microbiology, Centre for Laboratory Medicine, Tampere University Hospital, Tampere,* [3]*National Public Health Institute, Helsinki, and* [4]*Laboratory of Molecular Haematology, Department of Clinical Chemistry, Centre for Laboratory Medicine, Tampere University Hospital and University of Tampere Medical School, Tampere, Finland*

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Summary. We investigated responses to vaccination against pneumococcal polysaccharide, *Haemophilus influenzae* b (Hib) conjugate and tetanus toxoid antigens in 31 patients with chronic lymphocytic leukaemia (CLL) and 25 controls. While in the control group all antibody responses against different antigens were highly significant, in the patient group clear evidence for responsiveness was detected only in the case of Hib polysaccharide antigen. Certain CLL patient subgroups showed low reactivity against tetanus toxoid antigen. In conclusion, plain polysaccharide vaccines seem to be ineffective in patients with CLL. Conjugate vaccines, in turn, are immunogenic and may offer protection against infections caused by encapsulated bacteria in these patients. Further studies concerning an optimal vaccination scheme and clinical efficiency are warranted.

Keywords: CLL, *Haemophilus influenzae*, *Streptococcus pneumoniae*, vaccination, antibody response.

Chronic lymphocytic leukaemia (CLL) is the most common type of leukaemia in the western world. It is a clonal disorder characterized by the accumulation of small, mature-like, but functionally incompetent lymphocytes that are able to escape apoptosis. Infections are the major cause of mortality in patients with CLL and *Streptococcus pneumoniae* is the most important pathogen. Susceptibility to infections is often associated with immunodeficiency, the nature of which is complex and poorly understood; hypogammaglobulinaemia, IgG subclass deficiencies, functional abnormalities in T cells and different cytokines, an impaired capacity to produce specific immunoglobulins, and neutropenia are all conceivable components of the immunodeficiency seen in patients with CLL (Molica, 1994).

Vaccination is a straightforward option in raising immunity and reducing infections. However, immune responses to pneumococcal polysaccharide and influenza vaccines have been weak in patients with CLL, especially in those with advanced disease stage (Shaw *et al*, 1960; Jacobson *et al*, 1988; Gribabis *et al*, 1994). Conjugation of polysaccharides to protein carriers has rendered the polysaccharide antigen more immunogenic (Goldblatt, 2000). The efficacy of this type of conjugate vaccine has not, to our knowledge, been studied in patients with CLL.

In this study, we investigated the antibody responses to vaccination against different types of antigens, including those of pneumococcal polysaccharide, *Haemophilus influenzae* b (Hib) conjugate, and tetanus toxoid in patients with CLL.

Correspondence: Dr Marjatta Sinisalo, Department of Internal Medicine, Tampere University Hospital, PO Box 2000, FIN-33521 Tampere, Finland. E-mail: marjatta.sinisalo@tays.fi

PATIENTS AND METHODS

Patients and controls. The study population consisted of a cohort of 31 consecutive patients with CLL (22 men and nine women), aged 66 years (median, range 48–80 years), referred to the CLL outpatient clinic of Tampere University Hospital. Informed consent was obtained from all patients. The diagnosis and staging of CLL were based on standard clinical, morphological and immunophenotyping criteria, as described elsewhere (Aittoniemi *et al*, 1999). All patients had the B-cell phenotype. Disease stage according to Binet's classification was A in 14, B in eight and C in nine patients. The median duration of the disease was 3 years (range 0·5–14 years). At the time of vaccination, seven patients were on chemotherapy and 10 received steroids. Severe infections had been recorded in five patients and moderate infections in eight patients within a period of 2 years prior to

Table I. Medians and quartiles of antibody levels before vaccination, and proportionate changes in patients with CLL and controls.

	Pre-vaccination level median (quartiles)			Proportionate change median (quartiles)		
Vaccine antigen	CLL	Controls	*P*-value*	CLL	Controls	*P*-value*
Pneumococcal polysaccharide (μg/ml)	10·1 (2·92–24·6)	9·41 (6·03–15·0)	0·912	1·00 (0·80–1·35)	4·68 (3·25–7·70)‡	< 0·001
Serotype 1	0·77 (0·28–3·42)	1·13 (0·42–1·41)	0·956	1·00 (0·84–1·36)	3·81 (2·93–5·52)‡	< 0·001
Serotype 3	0·63 (0·27–2·39)	1·14 (0·63–1·42)	0·533	0·93 (0·79–1·19)	4·34 (2·49–7·93)‡	< 0·001
Serotype 6B	1·02 (0·33–2·64)	1·40 (0·68–3·06)	0·521	0·99 (0·75–1·88)	2·41 (1·30–4·25)†	< 0·001
Serotype 14	1·46 (0·45–2·87)	0·53 (0·31–1·27)	0·094	1·00 (0·83–1·29)	6·82 (1·37–21·6)‡	< 0·001
Serotype 19F	2·40 (0·77–8·30)	2·31 (1·39–3·81)	0·784	1·06 (0·73–1·40)	3·61 (2·46–10·1)‡	< 0·001
Serotype 23F	1·56 (0·33–3·91)	1·70 (0·73–2·91)	0·898	0·90 (0·75–1·12)	3·60 (1·54–7·27)‡	< 0·001
Haemophilus influenzae conjugate (μg/ml)	0·48 (0·22–3·06)	1·54 (1·02–3·71)	0·022	1·58 (0·99–5·47)†	42·1 (11·9–105)‡	< 0·001
Tetanus toxoid (IU/ml)	0·12 (0·01–1·03)	0·19 (0·01–3·61)	0·547	1·00 (0·88–2·00)	3·33 (1·39–72·1)†	0·005

*Mann–Whitney *U*-test.
The significance of proportionate increase between pre- and post-vaccination samples, Wilcoxon matched-pairs test, †$P < 0·05$; ‡$P < 0·001$.

vaccination (Aittoniemi *et al*, 1999). Hypogammaglobulinaemia (S-IgG < 6·7 g/l) was detected in 13 patients. Splenectomy had been performed in one case. For control purposes, 25 immunologically apparently healthy age- and sex-matched subjects with no haematological malignancy were included in the study.

Vaccines. Pneumococcal polysaccharide (Pnu-Immune 23, Wyeth Pharma, Munster, Germany), Hib conjugate (HibTITER, Wyeth Manufacturing, UK) and tetanus–diphtheria toxoid (Tetanus-d-rokote, National Public Health Institute, Helsinki, Finland) vaccines in 0·5 ml doses were used.

Vaccination and sampling schedules. If any of these vaccines had been given to the study subject within the last 5 years, no repeat vaccination was undertaken in order to avoid strong local reactions. All injections were given intramuscularly at separate sites during the same visit. Venous blood samples were taken before and 4 weeks after the vaccination. Post-vaccination blood samples were not obtained from two patients and one patient had been treated with intravenous gammaglobulin before the second blood sample was taken. These three patients were excluded from the study. Of the patients with CLL, 27 were included in the pneumococcal, 28 in the Hib and 18 in the tetanus part of the study. Of the controls, 25 were accepted for the pneumococcal and Hib parts, and 15 for the tetanus part of the study.

Determination of antibodies. IgG anti-capsular polysaccharide antibodies to pneumococcal serotypes 1, 3, 6B, 14, 19F and 23F, and IgG antibodies to Hib capsular polysaccharide were determined by enzyme immunoassay (EIA), as described elsewhere (Käyhty *et al*, 1995; Kurikka *et al*, 1995). The total pneumococcal anti-capsular polysaccharide antibody level was calculated as a sum of serotype-specific antibody levels. For the determination of IgG antibodies to tetanus toxoid, a slightly modified double antigen EIA was used (Kristiansen *et al*, 1997). As

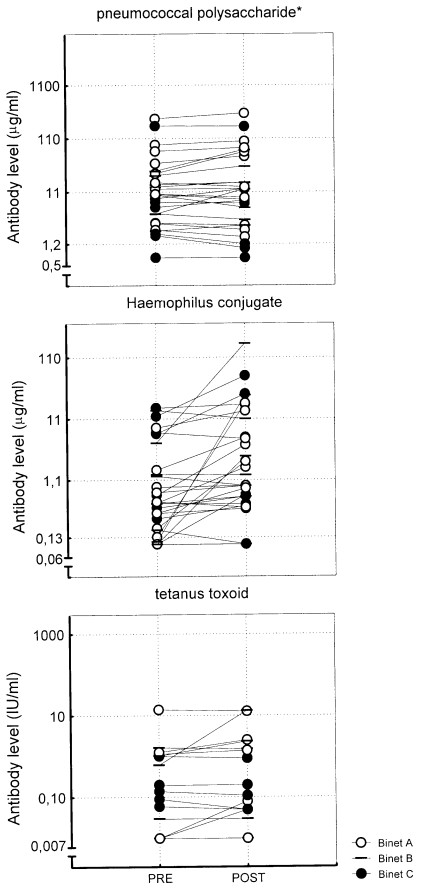

Fig. 1. Antibody responses to different types of antigens in patients with CLL. *expressed as a sum of serotype-specific antibody levels.

diphtheria toxoid worked as a carrier in the Hib conjugate preparation, specific antibody levels against diphtheria toxoid were not evaluated.

Statistical analysis. Pre-vaccination levels and proportionate changes between groups were compared using the Mann–Whitney U-test or the Kruskall–Wallis test, and the significance in antibody responses was evaluated using the Wilcoxon matched-pairs test. Proportionate changes had been calculated by dividing each patient's post-vaccination antibody level by that of prevaccination.

RESULTS

Pre-vaccination antibody levels and proportionate changes against different antigens in patients with CLL and controls are shown in Table I. The prevaccination antibody level against Hib polysaccharide antigen was lower in patients with CLL than in controls. The only significant response in the CLL patient group was observed in the case of Hib polysaccharide antigen: six (21%) out of 28 patients developed a protective antibody level ($\geq$ 1 μg/ml), and the post-vaccination antibodies were at the protective level in 54% of the patients (Käyhty *et al*, 1983). In controls, all responses were significant and more intense than those observed in patients with CLL.

Individual antibody responses against different types of antigens in patients with CLL are illustrated in Fig 1. When comparing the CLL patient subgroups with respect to Binet's class, total serum IgG and prevaccination antibody levels, the proportionate antibody response [median (quartiles)] to tetanus toxoid antigen was somewhat higher in those with Binet's class A [A: 2·00 (1·00–6·25), B: 1·00 (1·00–2·25) and C: 0·85 (0·79–0·88); $P = 0{\cdot}031$] and in those with normal serum IgG levels [IgG $\geq$ 6·7 g/l: 2·00 (1·00–2·25) and IgG $<$ 6·7 g/l: 0·88 (0·83–1·00); $P = 0{\cdot}021$]. A weak, albeit statistically significant, difference was also detected against pneumococcal polysaccharide antigen in patients with high prevaccination antibody levels ($>$ median) compared with those with low levels [1·17 (1·00–1·36) and 0·86 (0·74–1·27) respectively; $P = 0{\cdot}042$].

DISCUSSION

Antibody responses against different vaccination antigens were weak or lacking in patients with CLL compared with those observed in controls. This finding is in concordance with the results of earlier studies concerning the efficiency of pneumococcal polysaccharide, influenza and toxoid vaccines in patients with CLL (Shaw *et al*, 1960; Jacobson *et al*, 1988; Gribabis *et al*, 1994). The most significant response in CLL patients was detected against Hib polysaccharide antigen, the structure of which had been modified to a thymus-dependent form by conjugating it to a protein carrier (Goldblatt, 2000). Despite the relatively small number of patients in the tetanus toxoid vaccination group, low reactivity to this antigen could also be demonstrated in certain subgroups. Plain polysaccharides as thymus-independent antigens seemed to be ineffective in patients with CLL. Conjugate vaccines have proved to be highly immunogenic in patients with Hodgkin's disease (Chan *et al*, 1996). However, their efficacy has not previously been studied in patients with CLL. Our finding indicates that the Hib conjugate vaccine is more immunogenic than vaccines with plain polysaccharide antigen.

The response to Hib polysaccharide antigen was clearly lower in CLL patients than in controls. However, the relatively high proportion of patients with a protective antibody level points to the potential efficiency of conjugate vaccines against infections in these patients. The situation may be even better than antibody determinations alone would indicate: in contrast to plain polysaccharide vaccines, the conjugate vaccines are able to induce immunological memory, and protection has also been attributed to this circumstance (Goldblatt, 2000). As antibody responses may be further amplified by different vaccination strategies or adjuvants (Jurlander *et al*, 1995; Chan *et al*, 1996), efficacy may be further improved using an optimal vaccination scheme.

In conclusion, vaccine with plain polysaccharide antigen seemed to be ineffective in patients with CLL. Hib conjugate vaccine, for its part, was immunogenic even in patients with advanced disease stage or with hypogammaglobulinaemia. This immunogenicity was at least equal to or even better than that of a conventional toxoid protein vaccine. Judging by these findings, conjugate vaccines offer a better basis in the battle against infections caused by encapsulated organisms in patients with CLL. The next obvious step would be to study the immunogenicity and safety of pneumococcal conjugate vaccines in these patients. Further studies concerning an optimal vaccination scheme and clinical efficiency are warranted.

ACKNOWLEDGMENTS

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Short Communication

Haemophilus Influenzae Type b (Hib) Antibody Concentrations and Vaccination Responses in Patients with Chronic Lymphocytic Leukaemia: Predicting Factors for Response

MARJATTA SINISALO[a,*], JANNE AITTONIEMI[b], HELENA KÄYHTY[c] and JUHANI VILPO[d]

[a]*Department of Internal Medicine, Tampere University Hospital, P.O. Box 2000, FIN-33521, Tampere, Finland;* [b]*Department of Clinical Microbiology, Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland;* [c]*National Public Health Institute, Helsinki, Finland;* [d]*Leukemia Research Laboratory, Department of Clinical Chemistry, Centre for Laboratory Medicine, Tampere University Hospital and University of Tampere Medical School, Tampere, Finland*

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We have recently demonstrated a moderate vaccination response rate of 43% against *Haemophilus influenzae* type b (Hib) conjugate vaccine among adult and elderly patients with chronic lymphocytic leukaemia (CLL). We now investigated demographic and immunological factors predicting the favourable response and protective antibody concentrations for Hib conjugate vaccine in CLL. Lower age was associated with protective pre- and post-vaccination antibody concentrations. High IgG1 and IgA concentrations were also associated with the protective efficacy. High IgM, in turn, was the best predictor of a significant vaccination response. Again, lower age seemed to be involved in this outcome. Judging from these findings, it would seem beneficial to vaccinate all CLL patients with conjugate vaccines at the presentation of the disease. Investigations of a new pneumococcal conjugate vaccine in CLL are warranted.

Keywords: *Haemophilus influenzae*; Conjugate vaccine; CLL; Vaccination; Antibody response

INTRODUCTION

Patients with chronic lymphocytic leukaemia (CLL) are at increased risk of bacterial infections with encapsulated organisms such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib). These bacteria are often a cause of the sinopulmonary infections common in CLL patients [1,2].

CLL patients respond weakly to conventional polysaccharide vaccines [3,4]. Significantly higher response rates have been achieved with conjugate vaccines. In our recent study, a two-fold or better increase in Hib antibody concentration was achieved in 12 out of 28 CLL patients (43%) [4]. Even this response rate is low and unsatisfactory as compared to healthy control subjects, where the corresponding response rate was 96%. It would thus be important to know in advance which patients will benefit from vaccination. The aim of the present study was to investigate factors predicting the favourable vaccination response to Hib conjugate vaccine among patients with CLL.

PATIENTS AND METHODS

Patients and Controls

The study population consisted of 28 patients with CLL (20 males and 8 females), aged 66 years (median, range 48–80 years) admitted to the CLL outpatient clinic of Tampere University Hospital, and 25 healthy age- and sex-matched control subjects. All these patients and controls had participated in a previous vaccination trial concerning the efficiency of different types of vaccination antigens, and they had been vaccinated with one dose of Hib conjugate (HibTITER, Wyeth Manufacturing, UK), pneumococcal polysaccharide (Pnu-Immune 23, Wyeth Pharma, Munster, Germany) and tetanus–diphtheria

*Corresponding author. Fax: +358-3-2474388. E-mail: marjatta.sinisalo@tays.fi

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TABLE I Demographic and immunological characteristics of patients with CLL and controls. The values are expressed as medians (quartiles) if not otherwise stated

Parameter	Patients with CLL ($N = 28$)	Controls ($N = 25$)	p-Value
Sex (m/f)	20/8	15/10	0.402
Age (years)	66 (58–64)	59 (55–66)	0.069
S-IgG (g/l)	8.0 (5.3–9.9)	11.2 (9.5–12.6)	<0.001
S-IgG1 (g/l)	5.1 (3.0–6.5)	6.7 (5.7–8.2)	0.003
S-IgG2 (g/l)	1.73 (1.12–2.28)	3.37 (2.55–4.11)	<0.001
S-IgG3 (g/l)	0.22 (0.12–0.43)	0.25 (0.19–0.40)	0.232
S-IgG4 (g/l)	0.17 (0.06–0.40)	0.37 (0.23–0.75)	0.033
S-IgA (g/l)	1.17 (0.36–1.57)	2.12 (1.54–2.70)	<0.001
S-IgM (g/l)	0.31 (0.15–0.70)	1.01 (0.74–1.44)	<0.001
CD4 ($\times 10^9$/l)	1.30 (0.65–2.01)	0.77* (0.58–0.93)	0.027
CD8 ($\times 10^9$/l)	1.03 (0.62–1.95)	0.69* (0.51–0.83)	0.011
CD4/CD8	1.00 (0.82–1.75)	1.11* (1.00–1.56)	0.404

* $N = 22$.

toxoid (Tetanus-d-rokote, National Public Health Institute, Helsinki, Finland) vaccines [4]. Informed consent was obtained from all participants.

The diagnosis and staging of CLL were based on standard clinical, morphological and immunophenotyping criteria, as described elsewhere [5]. All patients had the B-cell phenotype. Disease stage according to Binet's classification was A in 13, B in 8 and C in 7 patients, and the median duration of the disease was three years (range 0.5–14 years). At the time of vaccination 7 patients were on chemotherapy and 10 received steroids (of those three received more than 0.3 mg/kg). Hypogammaglobulinaemia (S-IgG <6.7 g/l) was detected in 11 patients. Splenectomy had been performed in one case.

Determination of Antibodies

IgG antibodies to Hib capsular polysaccharide were determined by enzyme immunoassay (EIA) as described elsewhere [6,7]. Antibody concentrations >1 μg/l were considered protective as this concentration has been associated with long-term protection after vaccination with Hib polysaccharide vaccination [8]. The responses to vaccination were considered significant if the absolute increase was >1 μg/l and if increase was 2-fold or more.

Determination of Immunoglobulins and IgG Subclasses

Plasma concentrations of IgG, IgM, IgA and IgG subclasses were determined using a Behring Nephelometer (Behringwerke AG, Marburg, Germany) according to the manufacturer's instructions. CD4+ and CD8+ cell counts were measured by flow cytometry.

Statistical Analysis

Proportions were compared by Fisher's exact and median levels by Mann–Whitney U-test. Logistic regression analysis was applied to identify the independent effect of each parameter when appropriate.

RESULTS AND DISCUSSION

Demographic and immunological characteristics of CLL patients and controls are shown in Table I. Plasma IgG, IgG1, IgG2, IgG4, IgM and IgA concentrations were significantly lower in patients with CLL than in controls. Hence, many of the CLL patients were severely immunocompromised, which explains the poor overall vaccine response as compared to healthy control subjects [4]. Although the absolute count of CD4+ and CD8+ lymphocytes was higher in patients with CLL, no difference in CD4+ /CD8+ ratio was observed.

Results of logistic regression analysis identifying the independent effect of demographic and immunological findings on Hib antibody concentrations and responses are shown in Table II. Surprisingly, lower age was the most significant predictor with respect to protective anti-Hib antibody concentrations. High IgG1 and IgA concentrations were also associated with this effect.

A high IgM best predicted significant vaccination response. It can be speculated that the vaccination

TABLE II Results of logistic regression analysis identifying the independent effect of demographic and immunological findings on Hib antibody concentrations and response to vaccination. The results are expressed as medians (quartiles) if not otherwise stated

	Concentration of ≥1 μg/ml or significant response		
Parameter	Yes	No	p-Value
Pre-vaccination antibody concentration >1 μg/ml	$N = 10$	$N = 18$	
Age (years)	59 (55–66)	68 (63–71)	0.017
S-IgG1 (g/l)	6.1 (3.4–8.1)	4.5 (2.7–5.6)	0.048
Post-vaccination concentration >1 μg/ml	$N = 15$	$N = 13$	
Age	61 (55–67)	68 (66–70)	0.053
S-IgA (g/l)	1.29 (0.81–1.97)	0.89 (0.15–1.30)	0.062
Absolute increase >1 μg/ml	$N = 12$	$N = 16$	
S-IgM (g/l)	0.55 (0.22–0.78)	0.27 (0.13–0.34)	0.041
CD8 ($\times 10^9$/l)	0.99 (0.54–2.34)	1.14 (0.62–1.86)	0.064
Age	60 (56–67)	68 (64–71)	0.115
Proportionate increase >2×	$N = 12$	$N = 16$	
S-IgM (g/l)	0.55 (0.25–0.81)	0.25 (0.13–0.34)	0.052
Age	59 (56–67)	68 (64–71)	0.054

response to Hib conjugate vaccine resembles the primary humoral response, where IgM predominates or that the patient's IgM concentration is otherwise a good marker for immunocompetence in CLL. Neither disease stage according to Binet's classification nor hypogammaglobulinemia was a significant factor for response in our study. These results differ from those reported in recent study where patients who responded to Hib conjugate vaccine had more often less advanced disease state, normal immunoglobulins and higher total IgG and IgG2 and IgG4 levels than in non-responder group [9].

In conclusion, CLL patients with protective anti-Hib antibody concentrations and/or adequate response to Hib conjugate vaccine had higher IgM concentrations and were younger than those without response or protection. Neither disease stage nor hypogammaglobulinemia seemed to play any role. According to these findings it would appear to be beneficial to vaccinate all CLL patients with conjugate vaccine at the presentation of the disease. As pneumococcal infections are a more serious problem in patients with CLL, vaccination studies with the new pneumococcal conjugate vaccine are warranted.

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Similar Humoral Immunity Parameters in Chronic Lymphocytic Leukemia Patients Independent of V_H Gene Mutation Status

MARJATTA SINISALO[a,*], JANNE AITTONIEMI[b], TOMI KOSKI[c], GERARD TOBIN[d], ULF THUNBERG[e], CHRISTER SUNDSTRÖM[d], RICHARD ROSENQUIST[d], HELENA KÄYHTY[f] and JUHANI VILPO[c,g]

[a]*Department of Internal Medicine, Tampere University Hospital, Tampere, Finland;* [b]*Department of Clinical Microbiology, Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland;* [c]*Department of Clinical Chemistry, Centre for Laboratory Medicine, Tampere University Hospital, Tampere, Finland;* [d]*Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden;* [e]*Department of Oncology, Radiology and Clinical Immunology, Uppsala University, Uppsala, Sweden;* [f]*National Public Health Institute, Helsinki, Finland;* [g]*University of Tampere Medical School, Tampere, Finland*

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Chronic lymphocytic leukemia (CLL) is a clonal B-cell disorder, which has recently been divided into 2 subtypes based on the somatic hypermutation status of the immunoglobulin heavy chain (IgV_H) genes. In patients with unmutated tumor cells the survival time is approximately half of that in mutated cases, but the reason for this difference is poorly understood. Since infections are the major cause of mortality in CLL, we investigated the effect of the mutation status on host immunity and proneness to infections in patients with CLL. As expected, the disease progression seemed to be faster and the disease more advanced (Binet B and C) among unmutated patients than in the mutated ones. Surprisingly, no differences in humoral immunity [immunoglobulin G (IgG), IgM, IgA, IgG subclasses, anti-ABO blood group antibodies and mannan-binding lectin (MBL)] or immune responses (*Haemophilus influenzae* serotype b conjugate vaccination) were detected between these 2 patient groups. Furthermore, UM-patients were not more prone to infections compared to M-patients, and therapy had no impact on the incidence and pattern of infections in either of the patient groups. The current findings within this patient cohort reveal that the worse outcome in the unmutated subgroup is not caused by more severe defects in immunity and increased susceptibility to infections when compared with the hypermutated group. It is thus conceivable that active immunization procedures such as vaccination can successfully be applied on patients with unmutated IgV_H gene and advanced disease stage.

Keywords: CLL; Host immunity; Somatic hypermutation

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a clonal disorder characterized by the accumulation of small, mature-like, but functionally incompetent B lymphocytes. Recently, it has been divided into 2 different subtypes based on the hypermutation status of the variable region of the immunoglobulin heavy chain (IgV_H) gene [1,2]. In the unmutated form (UM-CLL), the malignant clone may emerge either from a naive B-cell or from a more mature cell, which have been activated through a germinal center (GC) independent pathway. The mutated form (M-CLL) may, in turn, derive from a B-cell that has undergone somatic hypermutation in a GC during an antigen response. In patients with UM-CLL, the average survival time is about half compared to those with M-CLL [2]. However, the reason for this difference is poorly understood. Large and complex chromosomal aberrations as well as poor prognostic chromosomal aberrations including 11q and 17p deletions have been observed to be more frequent in UM-CLL group than in M-CLL [3,4], which could associate with the worse outcome.

Infections are the major cause of mortality in patients with CLL [5,6]. Susceptibility to infections is often associated with immunodeficiency, and several components of immunodeficiency are seen in CLL patients

*Corresponding author. Address: Department of Internal Medicine, Tampere University Hospital, P.O. Box 2000, FIN-33521 Tampere, Finland. Fax: + 358-3-311 64388. E-mail: marjatta.sinisalo@pshp.fi

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including hypogammaglobulinaemia, IgG subclass deficiencies, functional abnormalities in T cells and different cytokines, an impaired capacity to produce specific immunoglobulins, and neutropenia [5,6]. However, the prevalence of immunodeficiency has not been currently studied in regard to IgV_H gene mutation status. In the present study, we have compared the concentrations of immunoglobulin (Ig) isotypes and mannan-binding lectin (MBL) between UM-CLL and M-CLL. Furthermore, for evaluation of the current state of immunocompetence we measured anti-ABO antibody levels and Haemophilus influenzae type b (HIB) vaccination responses, since a good vaccination response can be used as an indicator of a properly acting immune system [7].

PATIENTS AND METHODS

Patients

The study population consisted of 33 applicable CLL patients (25 males and 8 females), aged 67 years (median, range 48 – 79 years) enrolled from 2 consecutive patient series admitted to the CLL outpatient clinic of Tampere University Hospital [4,8]. The median time duration between the CLL diagnosis and the recruitment for the study was 3 years (range 0.5 – 14 years). The diagnosis and staging of CLL were based on standard clinical, morphological and immunophenotyping criteria, as described elsewhere [9]. All patients had the B-cell phenotype, and mature CLL, CLL/mix, or CLL/PL morphology. Disease progression rate was defined as slow (S), if the blood lymphocyte count increased less than 20% within a year, and fast (F), if the lymphocyte count was at least doubled within a year. Otherwise the progression rate was defined as intermediate (I). If the patient had received chemotherapy (chlorambucil) and/or steroids, the natural disease progression rate was not evaluable (9 patients). Severe infections (defined as infections needing hospitalization and intravenous antimicrobial treatment) had been recorded in 5 patients and moderate (defined as infections needing peroral antimicrobial treatment but no hospitalization) in 5 patients within a preceding period of 2 years. In 23 patients no or only mild infections had been detected. Each patient was graded only to 1 infection susceptibility group. Informed consent for the study was obtained from all patients.

V_H Gene Analysis

V_H gene PCR amplification followed by nucleotide sequencing was performed as described elsewhere [9]. V_H gene sequences deviating more than 2% from the closest aligned germline gene published in the GenBank, V-BASE or IMGT databases were defined as mutated (M), whereas sequences displaying less than 2% mutations were considered unmutated (UM).

Determination of Immunological Parameters

Plasma concentrations of IgG, IgM, IgA and IgG subclasses were determined using a Behring Nephelometer (Behringwerke AG, Marburg, Germany) according to the manufacturer's instructions. Serum concentration of mannan-binding lectin were measured using in-house enzyme immunoassay (EIA), as described elsewhere [10,11]. Plasma β_2-microglobulin concentration was determined by Cobas Core analyser using an enzyme immunoassay technique (Cobas Core β_2-microglobulin EIA, Hoffmann-La Roche, Switzerland) [12]. The anti-A and anti-B titres were determined applying standard tube technique for ABO grouping, as described elsewhere [13]. Antibody levels and responses against Hib conjugate vaccine (HibTITER, Wyeth Manufacturing, UK) were determined as described earlier [8]. Antibody levels > 1 μg/ml were defined as protective [14].

Statistical Analysis

Proportions were compared by Fisher's exact or chi-square, and levels by Mann–Whitney *U*-test.

RESULTS

Demographic characteristics of the CLL-patients with M-CLL and UM-CLL are shown in Table I. The disease progression seemed to be somewhat more progressive among patients with UM IgV_H genes compared to those with M, although the *p*-value did not quite reach the statistically significant level ($P = 0.085$). Furthermore, the UM patients belonged more frequently to Binet stage B or C class ($P = 0.031$), and tended to be more frequently anemic than the M patients ($P = 0.067$). However, UM-patients were not more prone to infections compared to M-patients, and therapy had no impact on the incidence and pattern of infections in either of the patient groups.

Immunological characteristics of the CLL-patients with M or UM IgV_H gene are shown in Table II. No differences in acquired (Ig isotype levels) or innate immunity (MBL level) were detected. Futhermore, no significant differences in HIB vaccination responses or anti-ABO IgM antibody levels were indicated between UM-CLL and M-CLL.

DISCUSSION

In this study we investigated whether the IgV_H gene mutation status has an effect on the clinical characteristics or host immunity in CLL. The disease progression seemed to be somewhat more progressive among patients with UM IgV_H genes compared to those with M, although the *p*-value did not quite reach the statistically significant level. Furthermore, the UM patients belonged

TABLE I Demographic characteristics of the CLL-patients with mutated or unmutated IgV_H gene

Parameter	Mutated CLLs ($N = 11$)	Unmutated CLLs ($N = 22$)	p-value
Sex (m/f)	9/2	16/6	0.687
Age, years (range)	64 (48–73)	68 (53–79)	0.174
Binet A/BC	8/3	7/15	0.031
FAB morphology (CLL)/(CLL/PL)/(CLL/mix)]	10/0/1	14/5/3	0.185
[a]Bone marrow infiltration (N/I/D/M)	1/3/3/4	0/6/8/8	0.536
Progression (slow or intermediate/fast)	7/2	7/10	0.085
Chemotherapy and/or steroid treatment	3/11	6/22	0.667
Infections (severe/moderate/mild or no)	1/1/9	4/4/14	0.563
Leukocyte count $\times 10^9$/l	95 (56–168)	105 (40–323)	0.499
Lymphocyte count $\times 10^9$/l	93 (46–142)	90 (36–310)	0.445
Hemoglobin g/l	130 (108–159)	117 (73–151)	0.126
Anemia (Hb $<$ 100 g/l)	0/11	6/22	0.067
Thrombocyte count $\times 10^9$/l	181 (98–258)	176 (22–453)	0.894
B_2-microglobulin (mg/l; $N = 28$)	3.3 (1.9–5.9)	3.9 (2.0–8.1)	0.252

[a]N, nodular; I, interstitial; D, diffuse; M, mixed

TABLE II Immunological characteristics of the CLL-patients with mutated or unmutated IgV_H genes. No significant differences were detected between the patient groups ($p > 0.05$). The levels are expressed as medians (quartiles) if not otherwise stated

Parameter	Mutated CLLs ($N = 11$)	Unmutated CLLs ($N = 22$)
IgG (g/l)	8.5 (6.8–13)	8.0 (3.1–15)
IgG1 (g/l)	6.0 (3.7–10)	5.4 (2.6–10)
IgG2 (g/l)	2.2 (0.63–6.5)	1.9 (0.33–3.7)
IgG3 (g/l)	0.28 (0.14–0.53)	0.36 (0.05–1.0)
IgG4 (g/l)	0.24 (0.041–1.5)	0.17 (0.005–1.5)
IgM (g/l)	0.27 (0.09–0.78)	0.37 (0.09–8.3)
IgA (g/l)	1.5 (0.31–3.1)	1.0 (0.27–4.5)
MBL (mg/l; $N = 32$)	2.9 (0.69–11)	4.6 (0.63–14)
Anti-ABO antibodies		
ABO blood group (A/B/AB/O)	4/1/1/3	7/2/5/7
Anti-A antibody titre ($N = 12$)	8 (4–8)	6 (2–16)
Anti-B antibody titre ($N = 19$)	4 (0–16)	3 (0–16)
Antibody response against HIB vaccination ($N = 16$)		
Pre-HIB (μg/ml)	0.70 (0.10–12.2)	0.49 (0.10–15.2)
Pre-HIB (> 1 μg/ml)	3/9	3/7
Post-HIB (μg/ml)	1.35 (0.60–55.3)	2.75 (0.40–186)
Post-HIB (> 1 μg/ml)	5/9	4/7
Absolute increase (μg/ml)	0.21 ($-$ 1.37–43.1)	$-$ 0.02 ($-$ 4.12–182)
Absolute increase (> 1 μg/ml)	3/9	3/7
Relative increase (ratio)	1.54 (0.79–12.5)	0.96 (0.70–244)
Relative increase ($>$ 2-fold)	4/9	3/7

HIB, Haemophilus influenzae serotype b

more frequently to Binet stage B or C class, and tended to be more frequently anemic than the M patients, which may be associated with the more advanced disease state. All these findings are in concordance with earlier studies [1,2]. However, UM-patients were not more prone to infections compared to M-patients. Furthermore, therapy had no impact on the incidence and pattern of infections in either of the patient groups.

No differences in acquired immunity (Ig isotype levels) or innate immunity (MBL level) were detected. Thus, no signs of increased humoral immunodeficiency among UM-CLL patients could be detected compared to M-CLL. In order to evaluate the current state of humoral immune function, we investigated the responses against HIB conjugate vaccine and measured anti-ABO blood group IgM antibodies in patients with CLL. However, no significant differences in HIB vaccination responses or anti-ABO IgM antibody levels were indicated between UM-CLL and M-CLL. Positive vaccination responses were seen in both UM-CLL and M-CLL patient groups, although the patients in the UM group belonged more often to Binet B and C classes.

In conclusion, no gross differences in humoral immunity or immune responses were detected between UM-CLL and M-CLL. This indicates that the worse outcome in UM CLL may be caused by faster

progression of the disease and not by defects in immunity and proneness to infections. We and others have previously shown that patients with CLL respond moderately to conjugate and toxoid vaccines and poorly to polysaccharide vaccines [8,15,16]. Thus, active immunization procedures like vaccination should also be applied on patients with unmutated IgV_H gene independently of the disease stage.

Acknowledgements

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Antibody response to 7-valent conjugated pneumococcal vaccine in patients with chronic lymphocytic leukaemia

Marjatta Sinisalo[a,*], Juhani Vilpo[b], Maija Itälä[c], Merja Väkeväinen[d], Jyrki Taurio[a], Janne Aittoniemi[e]

[a] *Department of Internal Medicine, Tampere University Hospital, Tampere, Finland*
[b] *Department of Clinical Chemistry, Centre for Laboratory Medicine, Pirkanmaa Hospital District, Tampere, Finland*
[c] *Department of Internal Medicine, Turku University Hospital, Finland*
[d] *National Public Health Institute, Helsinki, Finland*
[e] *Department of Clinical Microbiology, Centre for Laboratory Medicine, Pirkanmaa Hospital District, Tampere, Finland*

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Summary Chronic lymphocytic leukaemia (CLL) is a common adulthood mature B-cell neoplasm. Infections are the most important cause of mortality in this condition, and *Streptococcus pneumoniae* has been considered the most important single pathogen. We investigated the immunogenicity of 7-valent pneumococcal conjugate vaccine in patients with CLL. The study material comprised 52 patients with CLL and 25 age- and sex-matched controls. The subjects were vaccinated with Prevenar® pneumococcal conjugate vaccine. Serum samples were taken for antibody determinations before and four weeks after vaccination. Antibody response rates to vaccine antigens were lower in patients with CLL compared to controls. However, if the vaccine had been administered at an early stage of the disease, i.e. before commencement of chemotherapy and the development of hypogammaglobulinaemia, a significant vaccination response to at least six antigens was obtained in almost 40% of the CLL patients. Our results indicate that early administration of conjugate vaccine may be beneficial in CLL.

Introduction

Chronic lymphocytic leukaemia (CLL), a mature B-cell neoplasm, is the most common form of leukaemia. It affects individuals mostly in the fifth and sixth decades of life. Infections constitute the most important cause of mortality [1–3]; it is estimated that up to 60% of CLL patients will die due to infection [4]. Infections affect mainly the

* Corresponding author at: Department of Internal Medicine, Tampere University Hospital, P.O. Box 2000, FIN-33521 Tampere, Finland.
E-mail address: marjatta.sinisalo@pshp.fi (M. Sinisalo).

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respiratory tract [5], and *Streptococcus pneumoniae* (Pnc) is considered the most important single pathogen [2].

Susceptibility to infections in CLL arises from complex immunodeficiency, which appears at an early stage of the disease, preceding chemotherapy and the development of hypogammaglobulinaemia, and may occur long before the lymphoid system is overwhelmed by infiltration with CLL cells. B- and T-cell functions, representing adaptive (specific) immunity, are highly compromised [6—8], and patients are often hypogammaglobulinaemic, especially in the advanced stages of the disease [5,4,9]. Abnormalities in innate (non-specific) immunity including neutrophil and complement functions have also been described [10,11]. Furthermore, CLL patients evince defects in antigen presentation of the dendritic cells [12].

There are few reports of attempts to control immunodeficiency and susceptibility to infections in CLL. Antimicrobial prophylaxis increases resistance to antimicrobial agents. Intravenous immunoglobulin substitution, in turn, reduces only the occurrence of mild and moderate infections, but has no impact on severe infections and mortality, and is not considered cost-effective [13,14]. Since very little can be done to reinforce patients'innate immune system, efforts should focus on enhancing their own adaptive immunity.

Previous vaccination studies in patients with CLL have demonstrated that overall antibody responses to vaccines are weak, as reviewed by Sinisalo and colleagues [15]. Tetanus toxoid and *Haemophilus influenzae* serotype b (Hib) conjugate vaccines induce modest antibody responses, while in the case of Pnc polysaccharide vaccine no antibody response has been detected [16,17]. It is known that pneumococcal polysaccharides (PSs) are weak immunogens, especially if the immune system is suppressed. However, modification of PS antigens to a thymus-dependent, immunological memory-inducing form by conjugating them to protein carrier has rendered the bacterial PS antigens more immunogenic [18].

A 7-valent pneumococcal conjugate vaccine has recently been introduced. It carries capsular PSs of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F individually conjugated to diphtheria CRM197 carrier protein. Since Pnc is the most significant pathogen in CLL, we designed this trial to assess the immunogenicity of 7-valent pneumococcal conjugate vaccine in CLL patients and to compare the basic antibody concentrations and vaccine responses in leukaemic and non-leukaemic subjects.

Materials and methods

Patients and controls

The study population comprised a cohort of 52 consecutive patients with CLL (31 males and 21 females), aged 65 years (median, range 43—84 years), referred to the CLL outpatient clinics of Tampere and Turku University Hospitals. The control population comprised 25 age- and sex-matched subjects (median age 66, range 52—77, 16 males, and 9 females) without any known immunological or haematological defect admitted to the cardiac outpatient clinic of Tampere University Hospital. Since post-vaccination samples were unavailable in the case of 3 CLL patients and 1 control, 49 patients with CLL and 24 controls were included in the final study population. None of the patients or controls had obtained a pneumococcal polysaccharide or conjugate vaccine previously.

Informed consent to participate was obtained from all patients and controls. The study was approved by the ethical boards of Tampere and Turku University Hospitals and was conducted according to the Helsinki declaration.

Clinical and laboratory characteristics of the CLL patients are shown in Table 1. The diagnosis and staging of CLL were based on standard clinical, morphological and immunophenotyping criteria, as described elsewhere [19]. All patients had the B-cell phenotype. Disease stage according to classic Binet's classification was A (early stage of the disease, low risk) in 39, B (intermediate risk) in 9 and C (advanced stage, high risk) in 1 patients. The median duration of the disease was 2 years (range 0—21 years). At the time of vaccination only one patient was on chemotherapy (chlorambucil) and eight were receiving steroids for co-existing autoimmune disorders: idiopathic thrombocytopenic purpura (ITP) or autoimmune haemolytic anaemia (AIHA). Thirty-eight had never been treated for CLL, 11 had been on chemotherapy and autologous transplantation had been done in two of these. Severe infections (infections needing intravenous antibiotics and/or hospitalisation) had been recorded in five patients and moderate (infections with per oral antibiotics) in six within a period of one year prior to vaccination. Hypogammaglobulinaemia (S-IgG < 6.77 g/l) was detected in 14 patients. One patient had received radiotherapy because of splenomegaly, but none had been splenectomised.

Table 1 Clinical and laboratory characteristics of the patients with CLL

Character	Patients with CLL ($N = 49$)
Sex M/F	30/19
Age (years)	65 (43—84)
Disease duration (years)	2 (0.1—21)
Binet A/B/C	39/9/1
[a]Past or ongoing chemotherapy (yes)	11 (22%)
Ongoing steroid treatment (yes)	8 (16%)
Lymphocyte count ($\times 10^9\ l^{-1}$)	17.5 (0.9—339.8)
Platelet count ($\times 10^9\ l^{-1}$)	176 (70—581)
Hemoglobin (g/l)	137 (105—169)
IgG (g/l)	8.40 (3.68—16.69)
IgG1 (g/l)	6.40 (2.5—13.20)
IgG2 (g/l)	2.34 (0.69—5.53)
IgG3 (g/l)	0.34 (0.04—1.23)
IgG4 (g/l)	0.16 (0.00—1.70)
IgM (g/l)	0.40 (0.05—5.81)
IgA (g/l)	0.96 (0.25—4.56)

The values are expressed as medians and ranges, if not otherwise stated.

[a] Only 1 patient was on chemotherapy at the time of vaccination.

Vaccine

The vaccine used was 7-valent pneumococcal conjugate vaccine (Prevenar® in Finland, Wyeth Lederle Vaccines), which contains capsular PSs of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F individually conjugated to diphtheria CRM_{197} protein. The product is manufactured as a liquid preparation. Each 0.5 ml dose contains 2 μg of PS type 4, 9V, 14, 18, 19F, and 23F, 4 μg of PS type 6B (16 μg total PS), approximately 20 μg of CRM_{197} carrier protein, and 125 μg of aluminum phosphate as adjuvant.

Vaccination and sampling schedules

Patients and controls received one subcutaneous deltoid injection with Prevenar. Venous blood samples were taken before and four weeks after the vaccination. Serum was separated by centrifugation and stored at −20 °C.

Enzyme immunoassay (EIA) for anti-pneumococcal capsular PS antibodies

Serum IgG antibody concentrations to pneumococcal PSs 4, 6B, 9V, 14, 18C, 19F, and 23F were measured by EIA as previously described [20] with a 22F capsular PS adsorption step [21]. Briefly, microtitre plates (Maxisorp; NUNC, Roskilde, Denmark) were coated with 2.5—10 μg/ml of pneumococcal PS (American Type Culture Collection, Rockville, MD) in pyrogen-free phosphate-buffered saline (PBS). Controls and test sera were diluted 1:100 in PBS containing 10% foetal bovine serum (PBS-F), 10 μg/ml cell wall polysaccharide (CPS; Statens Serum Institute, Copenhagen, Denmark), and 30 μg/ml 22F capsular PS and incubated at +2 to 8 °C overnight. Serum pool 89-SF received from US Food and Drug Administration (Bethesda, MD) was used as reference and treated as above with 10 μg/ml CPS.

Results are given as micrograms per ml calculated on the basis of the assigned IgG values of the 89-SF reference serum [22]. The detection limits were: types 4 and 18C; 0.05 μg/ml, types 6B and 9V; 0.06 μg/ml, type 23F; 0.07, type 14; 0.12 μg/ml, and type 19F; 0.13 μg/ml, respectively. Antibody concentration >0.35 μg/ml was kept as suggestive pf a protection, since it is WHO recommended protective concentration for anticapsular antibodies to the seven serotypes in Prevenar applicable for assessing efficacy of pneumococcal conjugate vaccines against invasive pneumococcal disease in infants [23].

Statistical analysis

Antibody levels and proportionate changes between groups were compared by independent-samples *t*-test and the significance of responses within groups by paired-samples *t*-test. For the statistical analyses, the data distributions were normalised by log10-transformation. However, the values in the tables are expressed as true geometric mean concentrations (GMC) and 95% confidence intervals (CI) calculated through these transformations. Proportions deduced from antibody concentrations were compared by Pearson's chi-square test.

Results

Pre- and post-vaccination antibody concentrations

Pre- and post-vaccination antibody concentrations against pneumococcal polysaccharide antigens are shown in Table 2. No difference in pre-vaccination antibody concentrations was observed between CLL patients and controls. Among the patients with CLL, the advanced stage of disease (Binet B or C), past or ongoing chemotherapy, use of corticosteroids or history of increased susceptibility to infections were not associated with decreased pre-vaccination antibody concentrations. However, the antibody concentrations against certain serotypes (9V and 18C) were somewhat lower in CLL patients with hypogammaglobulinaemia compared to those with normal IgG level ($P = 0.011$ and $P = 0.031$, respectively). After vaccination, the antibody concentrations were significantly lower in CLL patients than in the controls for all serotypes.

Table 2 Pre- and post-vaccination antibody concentrations to pneumococcal polysaccharide antigens of 7-valent conjugate vaccine in patients with CLL and controls

Serotype	Pre-vaccination antibody concentration GMC (μg/ml) (95% CI)		*P*-value[a]	Post-vaccination antibody concentration GMC (μg/ml) (95% CI)		*P*-value[a]
	CLL (*N* = 49)	Controls (*N* = 24)		CLL (*N* = 49)	Controls (*N* = 24)	
4	0.13 (0.09—0.18)	0.11 (0.08—0.15)	0.535	0.32 (0.19—0.54)	1.64 (0.82—3.28)	<0.001
6b	0.30 (0.21—0.44)	0.35 (0.19—0.63)	0.704	0.63 (0.36—1.11)	4.44 (1.75—11.27)	<0.001
9v	0.37 (0.26—0.53)	0.33 (0.19—0.57)	0.738	1.12 (0.68—1.86)	7.63 (3.24—17.96)	<0.001
14	0.67 (0.47—0.94)	0.77 (0.44—1.33)	0.655	1.12 (0.69—1.82)	6.34 (2.96—13.58)	<0.001
18c	0.88 (0.61—1.27)	1.31 (0.75-2.27)	0.232	2.42 (1.49—3.92)	15.73 (8.14—30.41)	<0.001
19f	1.10 (0.77—1.56)	0.70 (0.44—1.11)	0.149	2.41 (1.48—3.94)	7.66 (3.23—18.18)	<0.001
23f	0.58 (0.39—0.86)	0.71 (0.40—1.28)	0.558	2.02 (1.14—3.56)	9.29 (4.08—21.13)	0.001

The concentrations are expressed as geometric means (GMC) with 95% confidence intervals (CI) prior to and one month after vaccination.
[a] Independent-samples *t*-test.

Table 3 Response rates[a], and the proportions of the pre- and post-vaccination antibody concentrations suggestive of protection (≥0.35 μg/ml[c]) to pneumococcal polysaccharide antigens of 7-valent pneumococcal conjugate vaccine in patients with CLL and controls

Serotype	Response rate[a] (N)		*P*-value[b]	Pre-vaccination concentration >0.35 μg/ml[c] (N)		*P*-value[b]	Post-vaccination concentration >0.35 μg/ml[b] (N)		*P*-value[b]
	CLL (*N* = 49)	Controls (*N* = 24)		CLL (*N* = 49)	Controls (*N* = 24)		CLL (*N* = 49)	Controls (*N* = 24)	
4	17 (35%)	20 (83%)	<0.001	7 (14%)	2 (8%)	0.467	24 (49%)	19 (79%)	0.014
6b	17 (35%)	18 (75%)	0.001	21 (43%)	13 (54%)	0.363	28 (57%)	20 (83%)	0.027
9v	21 (43%)	21 (88%)	<0.001	28 (57%)	11 (46%)	0.363	37 (76%)	22 (92%)	0.100
14	10 (20%)	18 (75%)	<0.001	34 (69%)	18 (75%)	0.619	35 (71%)	21 (88%)	0.127
18c	19 (39%)	20 (83%)	<0.001	40 (82%)	22 (92%)	0.260	43 (88%)	24 (100%)	0.074
19f	21 (43%)	21 (88%)	<0.001	41 (84%)	18 (75%)	0.377	45 (92%)	23 (96%)	0.525
23f	23 (47%)	19 (79%)	0.009	27 (55%)	17 (71%)	0.197	38 (78%)	22 (92%)	0.139

[a] Proportionate increase at least two-fold and post-vaccination level at least 0.35 μg/ml.
[b] Chi-square test.
[c] WHO recommended protective concentration for anticapsular antibodies to the seven serotypes in Prevenar applicable for assessing efficacy of pneumococcal conjugate vaccines against invasive pneumococcal disease in infants.

Antibody responses

Response rates and the proportions of pre- and post-vaccination antibody concentrations suggestive of protection to pneumococcal polysaccharide antigens of the conjugate vaccine in patients with CLL and controls are shown in Table 3. A significant increase in antibody concentrations was detected in both groups for all antigens after vaccination ($p < 0.05$). However, the change was significantly higher among the controls, varying from 8.3- to 23-fold depending on the antigen, compared to the 1.7- to 3.5-fold change in patients with CLL ($p < 0.005$). A significant response — defined as an at least two-fold increase and a post-vaccination concentration of at least 0.35 μg/ml — was observed in 20—47% of the CLL patients depending on serotype. In controls the percentage varied from 75 to 88%. In the patients with CLL, the proportions of antibody concentrations suggestive of protection (>0.35 μg/ml) rise from 14—84% to 49—92% after vaccination, depending on the serotype while in controls the same percentages were 8—92% and 79—100%, respectively. The use of corticosteroids or a history of infections had no effect on response frequencies in patients with CLL. However, significant response rates were lowered in the case of certain serotypes in the subgroups of patients with advanced disease (Binet class B/C; serotypes 4, 9V), past or ongoing chemotherapy (serotypes 4, 6B, 9V, 18C) and hypogammaglobulinaemia (serotypes 9V, 18C, 19F) ($p < 0.05$).

The distributions of the number of significant responses in individual patients with CLL and controls are shown in Fig. 1. Among the controls, a significant response to at least six antigens was achieved in 71% of cases (17/24), while in the CLL patients the percentage was 24% (12/49) ($P < 0.001$; Fig. 1). However, if the vaccine had been administrated at an early stage in the disease (Binet A), before commencement of chemotherapy and the development of hypogammaglobulinaemia, 39% (11/28) of these CLL patients developed a significant response to at least six antigens, while in other patients the figure was 5% (1/21) ($P = 0.007$).

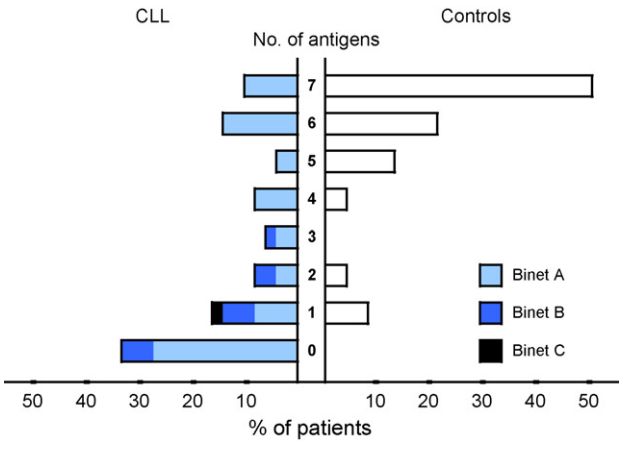

Figure 1 Distribution of the number of significant responses (proportionate increase at least two-fold and post-vaccination concentration at least 0.35 μg/ml) against pneumococcal vaccine serotypes in individual patients with CLL and controls.

Discussion

It is well established that infections are the most prominent cause of mortality in CLL: as many as 60% of CLL deaths may be attributable to infections [4]. *S. pneumoniae* has been considered the most important single pathogen involved in these cases [2].

Here we demonstrated that pre-vaccination anti-pneumococcal antibody concentrations were very similar in patients with CLL and controls. Since immunological defects and poor humoral response probably precede the diagnosis of CLL by several years in the manner of premalignant state, known as monoclonal B-cell lymphocytosis (MBL) [24], this finding raises questions as to the aetiology and function of these antibodies in CLL patients. This subject should be further studied.

Earlier investigations have demonstrated that the traditional unconjugated pneumococcal polysaccharide vaccines do not raise a significant immune response in CLL patients [16,17,25,26]. Promising results have, however, been obtained when polysaccharide antigens have been modified to a thymus-dependent, memory-inducing form by conjugating them to an immunogenic protein carrier as exemplified by Hib conjugate vaccine [27,28]. Here we have taken the next logical step, i.e. tested the immunogenicity of protein-conjugated pneumococcal antigens themselves. The results clearly demonstrate that significant responses against most tested antigens are frequently obtained by using novel conjugate vaccines. The proportions of antibody concentrations suggestive of protection after vaccination seemed also to be relatively high, though the efficacy of this protective concentration has been determinated only in infants against invasive pneumococcal disease, and it is probably not sufficient in adults [23].

Furthermore — according to the study of Robinson and colleagues [29] made in the United States — the serotype coverage of the 7-valent conjugate vaccine against invasive pneumococcal infections in patients $\geq$65 years was only 56% compared to that of 86% in 23-valent polysaccharide vaccine. Thus, active research for improved vaccine formulations in this age group is urgently needed. The two approaches are the development of conjugate vaccines with higher valency, including the significant serotypes in elderly population, and evaluation of different protein antigens common to all pneumococcal serotypes as potential vaccine candidates.

Antibody concentration changes and significant response rates were markedly lower among patients with CLL than in controls. The response rates were further compromised by advanced disease state, chemotherapy and hypogammaglobulinaemia, the rates varying from 20 to 47%, and only 24% of CLL patients mounted a significant response to at least six antigens. This is in accord with our earlier findings concerning Hib conjugate vaccine, where 21% of CLL patients developed a significant response [17]. However, if the vaccine had been administered at an early stage in the disease, before commencement of chemotherapy and the development of hypogammaglobulinaemia, almost 40% of the patients developed a significant response to at least six antigens. Furthermore, the antibody responses induced by the conjugate vaccine in this study were superior compared to those in our earlier studies with pneumococcal polysaccharide vaccine in CLL [17]. Optimal dosing of conjugated vaccines for adults [30], use of novel adjuvants, and boosting either with conjugate or PS vaccine [31] might further improve the immunogenicity of the conjugate vaccines in CLL patients.

In conclusion, antibody response rates of pneumococcal conjugate vaccine were lower in patients with CLL compared to controls. However, if the vaccine had been administered at an early stage in the disease, before initiation of chemotherapy and the development of hypogammaglobulinaemia, a significant vaccination response was obtained in almost 40% of CLL patients. Thus, especially since the conjugate vaccine can induce long-lasting, thymus-dependent immunity against the pathogen — vaccination of CLL patients at the time of diagnosis may be warranted.

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