Antibody responses to vaccination and immune function in patients with haematological malignancies

Studies in patients with chronic lymphocytic leukaemia and autologous stem cell transplant recipients

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De vorming van antistoffen na vaccinatie en de immuunstatus bij patiënten met een hematologische maligniteit

Studies bij chronische lymfatische leukemie en na autologe stamceltransplantatie

(met een samenvatting in het Nederlands)

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CHAPTER 1

General introduction

Patients with haematological malignancies frequently suffer from immunodeficiency as a consequence of the disease itself or as a consequence of therapy. In chronic lymphocytic leukaemia (CLL), hypogammaglobulinaemia and other immunological abnormalities are present, leading to an increased infection risk with significant morbidity and mortality[1,2]. During the last decades, increasing numbers of patients with various diseases have undergone autologous stem cell transplantation (aSCT). After recovery from the neutropenic phase, immune function may remain impaired for a prolonged period[3], leading to an increased risk for infections. Vaccination is an option to improve specific adaptive immunity in order to reduce susceptibility to infections. Immune function, the risk of infection and responses to vaccination will be discussed in patients with CLL and autologous stem cell transplant recipients. Normal immune responses in healthy adults are briefly discussed.

Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia (CLL) is the most common type of leukaemia, accounting for over 25% of adult patients with leukaemia seen in clinical practice[4]. The clinical course of CLL is highly variable with prognosis depending on stage of the disease. Over three decades ago, Rai developed a clinical staging system for CLL based on the presence of lymphocytosis, lymphadenopathy, anaemia and/or thrombocytopenia, and enlarged liver and/or spleen[5]. The median survival times range from 19 months for Rai stage IV to >150 months for Rai stage 0. This clinical staging system, or the comparable Binet staging system[6], is nowadays still in use. However, 50% of patients in the low-risk group (Rai stage 0 or Binet stage A) eventually need treatment[7]. New prognostic factors including cytogenetic abnormalities (13q deletion, trisomy 12) and the mutational status of Ig genes have been defined[8,9]. Since sequencing of Ig genes is costly and not widely available, surrogate markers for Ig gene mutation profiles are needed. Crespo *et al* analysed the expression of the 70-kDa zeta-associated protein (ZAP-70) in patients with CLL. They found a correlation between the expression of ZAP-70 (measured by flowcytometry, immunohistochemistry or Western blotting) and the Ig genes mutational status[10]. Furthermore, flowcytometric determination of ZAP-70 has been proven to be accurate in predicting outcome in patients with CLL[11,12]. Clinical staging systems and the new prognostic factors are supposed to be complementary in predicting prognosis[13].

CLL is a monoclonal proliferation of lymphocytes, mostly of the B-cell lineage[14]. For many years, humoral immunodeficiency, reflected in hypogammaglobulinaemia, has been considered the cause of this increased infection risk[15]. However, also patients with normal serum immunoglobulin levels may suffer from recurrent infections, whereas some patients with hypogammaglobulinaemia do not have recurrent infections. Despite having marked and persisting hypogammaglobulinaemia, patients responding to treatment of the leukaemia tend to have fewer and less serious infections[16,17]. More recently, defects in cellular immunity including deficient T helper cell function and natural killer cell activity in patients with CLL have been described[18,19]. T helper cells are thought to be intrinsically normal, but the predominance of malignant B cells as accessory cells down regulates the function of T cells through production of soluble factors that have not been identified in detail[20,21]. Additionally, decreased levels of serum complement proteins (both classic and alternative pathway components) and low haemolytic activity of the classic complement pathway have been reported in patients with CLL[22,23]. Defects in the complement system correlates with disease stage with a prevalence of 40% in early stage disease (Rai stage O-I), increasing to 100% in more progressive disease stages (Rai II-IV)[22].

Patients with CLL have a predisposition to infections: approximately 50 – 60% of patients with CLL will die from infections including pneumonia, urinary tract infections, sepsis and soft tissue infections[24]. Causes of infections are often encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. Influenza virus can, however, also lead to serious morbidity and even mortality in patients with CLL. Vaccinations with influenza virus vaccine, pneumococcal polysaccharide vaccine and *Haemophilus influenzae* type b (*Hib*) conjugate vaccine are recommended in patients with CLL and other haematological malignancies[25,26]. However, antibody response rates are often low, and only 30 - 50% of vaccinated patients show a sufficient response[27,28]. In order to improve antibody responses, administration of ranitidine in patients with CLL during vaccination with *Hib* and pneumococcal vaccines has been studied. Ranitidine exerts immunomodulatory effects by antagonising histamine receptor type 2. Significantly better antibody responses to tetanus toxoid (TT) and *Hib* conjugate vaccine were found during ranitidine treatment[29,30]. Other strategies, including booster vaccinations and the use of conjugated vaccines for capsular polysaccharides, have also been used in order to improve response rates with variable results[27,30].

Autologous stem cell transplantation

Immune reconstitution after aSCT

High dose chemotherapy followed by autologous peripheral stem cell transplantation (aSCT) is a widely used therapy for many haematological malignancies[31,32]. Peripheral stem cell transplantation is believed to shorten the period of severe neutropenia compared to bone marrow transplantation[33], but parts of the immune function remain impaired for a year or even longer after transplantation[34-36]. Recovery of the innate immunity is usually seen within weeks after aSCT[34-36]. For instance, numbers of natural killer cells normalise within 2-4 weeks after transplantation[37]. On the contrary, adaptive immunity takes much longer to recover. Reconstitution of B and T lymphocytes show characteristic kinetics. B lymphocytes reach normal levels approximately 3-6 months after aSCT[35,36]. The immunoglobulin V-gene repertoire in autologous transplant recipients initially resembles that found in the neonate by having less somatic mutations compared to normal adults[38]. The phenotype of B lymphocytes also resembles that of infants[39,40]. B-lymphocyte reconstitution therefore appears to recapitulate normal ontogeny although with different kinetics.

T lymphocytes regain their function more slowly than B lymphocytes. High dose chemotherapy will destroy most T cells in the patient. If non-T cell depleted grafts are used, some mature T cells will be present in the graft. In addition, stem cells will develop into mature T cells following normal ontogeny including maturation in the thymus[35,36]. Thymus function in aSCT patients is reduced because of age and previous chemotherapy and therefore, the maturation of T cells will take a long time. The number of $CD8^+$ T cells normalises relatively early after aSCT compared to $CD4^+$ T cells[41-43]. Consequently, a decreased CD4/CD8 ratio is found until at least one year after aSCT. A possible explanation for this finding is that thymic as well as extrathymic lymphopoiesis appears important for the generation of $CD8^+$ cells, whereas no extrathymic pathways for the generation of $CD4^+$ cells have been identified[44]. Additionally, peripheral expansion of mature $CD8^+$ T cells, which remained in the secondary lymphoid tissue after chemotherapy, can contribute to the relatively early normalisation of $CD8^+$ T cell numbers[45]. $CD4^+CD45RO^+$ cells ("memory" helper T lymphocytes) reach normal levels within months after transplantation, whereas CD4 $CD45RA^+$ cells ("naive" helper T lymphocytes) remain low for a longer period[46-48]. The mature $CD4^+CD45RO^+$ cells probably represent mature T cells in the graft, or, alternatively, mature recipient T

cells that survived the conditioning regiment.

Functional cellular immunity in terms of T-cell proliferation is also compromised during the first year after aSCT[46,49]. For instance, T-cell proliferation in response to the mitogenic stimulant PHA was significantly decreased during the first year after aSCT in breast cancer patients[49]. Data on cytokine production after aSCT as measurement of cellular immunity however are limited and often restricted to the early post-transplantation period only[50-52].

Infections after autologous stem cell transplantation
Previous chemotherapy and the conditioning regimens for aSCT lead to impairment of the immune system and predispose patients to infections, not only during the recovery phase but also for a longer period after white blood cell reconstitution[53,54]. Although infectious complications are less frequent than in allogeneic transplantation, they are also a major source of morbidity in patients undergoing aSCT. High incidences of bacteraemia caused by gram-positive bacteria are found after aSCT[55,56]. These studies were, however, confined to the immediate post-transplant period. Data regarding infectious complications during the first year after aSCT are sparse. Crippa *et al* studied infectious complications during the first 100 days in patients receiving either CD34-selected or non-selected aSCT[57]. Bacterial infections were found in 34 and 16%, respectively, and consisted predominantly of bacteraemia (other than due to coagulase-negative staphylococci). This study concerned infections mainly in the early period after transplantation, including the neutropenic phase and the period of use of intravascular catheters. In a study by Engelhard *et al*, both early (< 100 days after transplantation) and late (> 100 days after transplantation) infectious complications were studied in patients receiving allogeneic or autologous bone marrow or peripheral stem cell transplantation[58]. Of 2392 patients receiving autologous stem cell or bone marrow transplantation, 11 (0.46%) developed late invasive pneumococcal infection (IPI). Two of these patients (18%) died because of invasive pneumococcal disease. In developed countries, the annual incidence of invasive pneumococcal infection varies between 0.5 per 1000 in persons aged ≥65 years and 0.05 per 1000 in the non-infant, non-elderly general population[59]. Thus, the incidence of 4.6 per 1000 patients receiving autologous transplantation is high (relative risk 9.2 compared to the elderly). Therefore, preventive measures such as vaccination are required in this group of patients.

Vaccination after aSCT

Immunity to recall antigens declines after stem cell transplantation[60-63]. Because of the increased risk for infections, vaccinations with pneumococcal vaccine and *Hib* vaccine have been recommended by institutions such as the Centers for Disease Control and Prevention, the American Society of Hematology and the European Group for Blood and Marrow Transplantation[64-66]. In the European guidelines, meningococcal vaccination is not routinely recommended but advised to be considered in situations where the risk of meningococcal disease is increased[65]. All guidelines apply to both allogeneic and autologous peripheral stem cell as well as bone marrow transplantation.

Over the years, polyvalent pneumococcal polysaccharide vaccines have been developed. Currently, the 23-valent polysaccharide vaccine contains 25 µg of purified capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. These serotypes cause 80 - 90% of all IPI in the general population[67,68]. The 23-valent pneumococcal polysaccharide vaccine, however, elicits a T-cell independent immune response. Because of the slow reconstitution of the immune response to T-cell independent antigens after aSCT, these conventional polysaccharide vaccines result in disappointingly low antibody responses[63,69-72]. Recently, a heptavalent conjugated pneumococcal polysaccharide vaccine consisting of capsular polysaccharides of pneumococcal serotype 4, 6B, 9V, 14, 18C, 19F and 23F has been developed. In Western countries, these 7 serotypes are responsible for 70 - 88% of IPI's in young children under 5 years of age but less than 50% in older age groups[73]. In this vaccine, the pneumococcal antigens are covalently linked to a carrier protein. Conjugated polysaccharide vaccines induce a T-cell dependent antibody response and have been proven to be immunogenic in young children[74-77]. In children, priming with a conjugated vaccine boosts the subsequent response to polysaccharide-based vaccination for the serotypes in the conjugate vaccine[78]. Post-transplantation vaccination schedules consisting of conjugated *Hib* vaccine and conjugated pneumococcal polysaccharide followed by non-conjugated pneumococcal vaccines have also been recommended in stem cell transplant recipients[64,65,79]. Indeed, vaccination with conjugated *Hib* vaccine in patients with haematological malignancies leads to adequate anti-*Hib* antibody responses. However, antibody responses to pneumococcal polysaccharide vaccines, even in conjugated form, were generally less[69,70,79].

Antibody titre as determined by enzyme linked immunoassay is a generally accepted surrogate marker of clinical effectiveness of vaccination. However, determination of functional parameters such as avidity and phagocytosis-inducing capacity of antibodies can provide additional information regarding the effects of vaccination[80-83]. For instance, during the first year after *Hib* vaccination, a decrease in antibody levels with a concurrent increase in antibody avidity has been described in children[81]. Higher antibody avidity has been shown to improve bactericidal activity in vitro[84]. Phagocytosis-inducing capacity of antibodies has been studied in allogeneic bone marrow transplantation. Following transplantation, opsonophagocytosis-inducing capacity of the antibodies against *Streptococcus pneumoniae* initially declined, whereas vaccination with a 23-valent non-conjugated pneumococcal polysaccharide vaccine post-transplantation resulted in an increase of the opsonophagocytic activity[85]. Another study determined serum opsonic activity against *Streptococcus pneumoniae* serotype 6A in allogeneic BMT recipients with pneumococcal infection[86]. Two patients with sufficient anti-pneumococcal antibody levels had impaired opsonic activity. A thorough analysis of the immune response to vaccination should also include functional parameters such as antibody avidity or opsonophagocytosis-inducing capacity. However, laboratory tests for avidity and opsonophagocytosis are only available in a research setting and, therefore, less suitable for routine use in a clinical setting.

The normal immune response

The normal immune response to micro-organisms comprises the innate (formerly known as non-specific immunity) and acquired (or specific) immune system. The innate immune response is a complex defence mechanism that involves opsonisation, phagocytosis and lysis of pathogens and infected cells by macrophages, neutrophils and natural killer cells[87-89]. An overview of important components of the innate immune response is shown in *Figure 1*.

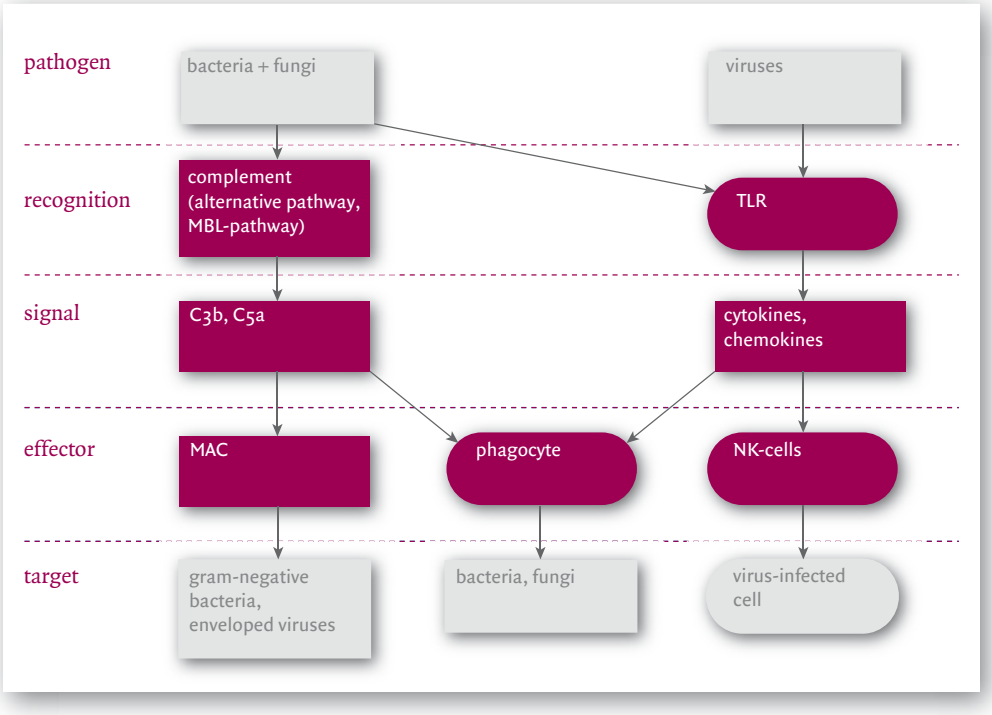

Figure 1. Important pathways of the innate immune response. MAC: membrane attack complex. MBL: mannose binding lectin. NK cells: natural killer cells. TLR: Toll-like receptor.

A spectrum of proteins and peptides, such as lactoferrin and beta-defensins, are present in blood and other body fluids with potent antimicrobial activity. Additionally, the complement-mediated alternative and mannose-binding lectin (MBL) pathways are important innate defence mechanisms against various pathogens[90,91]. After breaching the mechanical barrier of epithelium of skin or mucosal tissue, pathogens are recognised by pathogen recognition receptors (PRRs) that are present as soluble factors such as MBL, or on various cells of the innate immune system (on natural killer (NK) cells, macrophages and dendritic cells)[92]. These PRRs bind to structurally conserved pathogen-associated molecular patterns (PAMPs). MBL is a serum protein of the collectin family that, after binding to PAMPs, initiates the lectin pathway of complement activation[90]. Toll-like receptors (TLR) are transmembrane PRRs that detect bacterial lipoproteins and lipoteichoic acids (TLR2) and lipopolysaccharide (LPS, detected by TLR4).

After ligand binding to TLR (involving also co-receptor CD-14), an intracellular signalling pathway is activated that results in exposure of the nuclear translocation domain of transcription of nuclear factor-κB (NF-κB)[89,93]. NF-κB subsequently activates transcription of pro-inflammatory genes, leading to production of cytokines and chemokines that activate phagocytes and NK cells. Eventually, phagocytosis and lysis of pathogens and infected cells takes place (*Figure* 2).

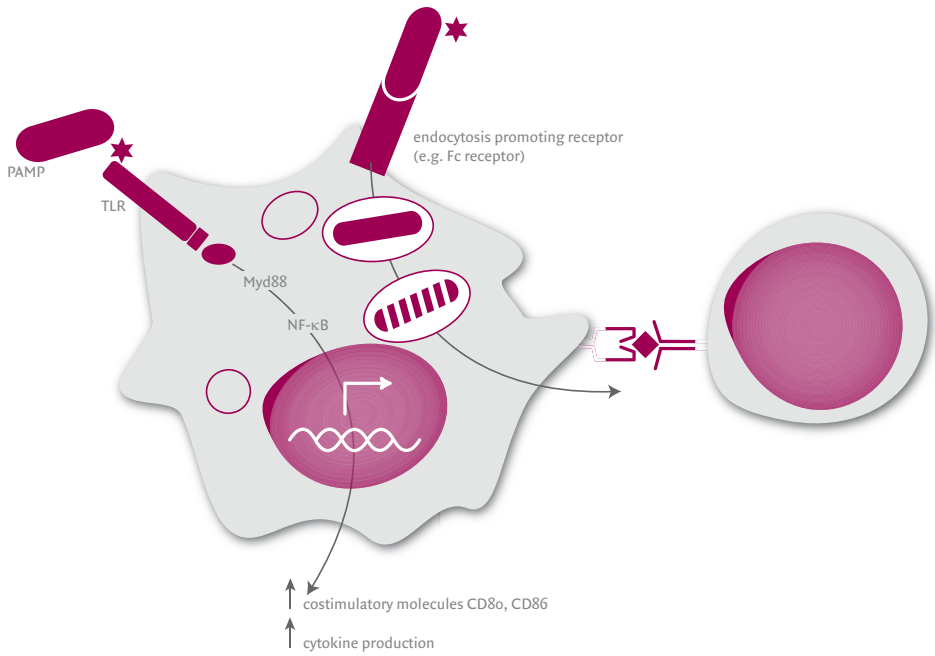

Figure 2. TLR-signalling.

The innate immune response is a fast-acting response that develops early in ontogeny but has no immunological memory[94]. In contrast, the acquired immune system is present at birth and develops during the first years of life and includes a cellular and humoral pathway. In the cellular pathway, antigen-presenting cells (APC) present peptide antigens in a complex with a major histocompatibility complex (MHC) molecule to T lymphocytes. Cytotoxic effector T

lymphocytes ultimately kill viral infected cells whereas helper T lymphocytes will secrete cytokines and activate other effector cells[94]. Humoral immunity involves recognition of antigen by B lymphocytes and stimulation of B lymphocytes by cytokines produced by T lymphocytes as well a co-stimulation by complement components[95,96]. This results in proliferation and differentiation of B lymphocytes and eventually production of specific antibodies. These antibodies subsequently bind to the antigen, a process called opsonisation. After binding of the Fc portion of the antibody to receptors on phagocytes, the antibody-antigen complex is internalized by the phagocytes. In case of T-cell involvement like in T-cell dependent antibody responses upon protein vaccine antigens or polysaccharide-protein conjugate vaccines, not only antibody secreting B lymphocytes but also memory B lymphocytes will develop. Stimulation of these memory cells by antigen will lead to a secondary antibody response[94].

Humoral immune responses are classified as either T-cell dependent (TD) or T-cell independent (TI). In TD antibody responses, the protein antigen is bound to the immunoglobulin receptor on B lymphocytes and internalised. After processing, antigenic peptides are presented by MHC class II molecules on the surface of the B lymphocytes (*Figure 3*). The peptide-MHC complex in combination with co-stimulatory signals like CD40-CD40L interaction, subsequently activates T lymphocytes to differentiate into effector cells, producing IFN-γ and IL-2 (T helper 1 (Th1) cells) or IL-4, IL-5 and IL-13 (T helper 2 (Th2) cells)[94]. Th1 cytokines are important in cell-mediated immune responses, whereas Th2 cytokines are required for B-lymphocyte proliferation and differentiation into plasma cells.

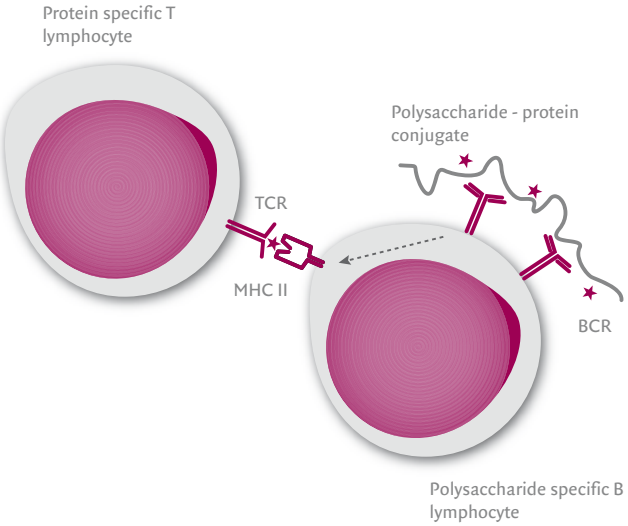

Figure 3. T-cell dependent immune response to a polysaccharide-protein conjugate.

Encapsulated bacteria such as *Haemophilus influenzae* type b (*Hib*) and *Streptococcus pneumoniae* have capsules consisting of polymers of oligosaccharide repeating units[97]. The antibody response to capsular polysaccharides does not require the help of T cells; these antigens are, therefore, classified as T-cell independent (TI) antigens (*Figure 4*)[98].

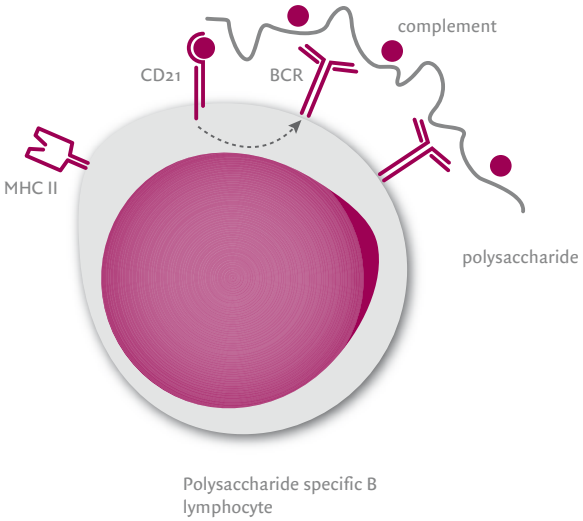

Figure 4. T-cell independent immune response. By cross-linking B-cell receptors, polysaccharide antigen activates B lymphocytes independent of T lymphocytes.

The magnitude of the antibody response can however be augmented by T cells. Polysaccharides are therefore classified as TI-2 antigens, in order to distinguish them from lipopolysaccharides, which are truly independent of T-cell help (TI-1). B-lymphocyte activation by polysaccharides involves co-cross-linking of B-cell receptors with CD21 (see *Figure 4*). Polysaccharides can activate the complement system, which results in deposition of C3d on the polysaccharide. On polysaccharide specific B lymphocytes, the B-cell receptor binds the polysaccharide epitopes while CD3d binds to CD21[99]. TI-2 antibody responses result in low affinity antibodies, mostly of the IgG2 and IgM isotype, without the induction of memory B lymphocytes. TI-2 antibody responses, and thus the response to polysaccharides, are poor in immature immune systems such as seen early in life or in patients with haematological malignancies[100,101].

Aims and outline of the thesis

This thesis addresses immune function and reconstitution and improvement of antibody responses in patients with haematological malignancies, with emphasis on patients with CLL and autologous stem cell transplant recipients.

The first part concerns immune responses to vaccination in patients with CLL. Booster vaccination with influenza virus vaccine was used to improve the rather poor immune responses in patients with CLL **(chapter 2)**. Patients with CLL are at increased risk for infections with encapsulated bacteria. Vaccination with *Hib* or pneumococcal vaccine leads to relatively low response rates. Ranitidine, a histamine receptor antagonist, has various immune modulatory properties. The role of ranitidine in the improvement of immune responses to protein and polysaccharide vaccines in patients with CLL is studied in **chapter 3**.

The second part of this thesis describes immune function in autologous stem cell transplant recipients. Because of the increased susceptibility of these patients to infections, revaccination after transplantation is recommended. No standard protocol regarding (re)vaccination after autologous stem cell transplantation exists and vaccination schedules differ significantly amongst different transplantation centres. Therefore, we conducted the Vaccination after Autologous Stem cell Transplantation (VAST) trial. Patients who underwent autologous stem cell transplantation were included in a vaccination protocol consisting of three vaccination rounds with DTP (diphtheria, tetanus and polio), *Hib* and pneumococcal vaccine. Aim of this study is to analyse the immune response to vaccination and to establish a vaccination schedule for patients who underwent autologous

stem cell transplantation. **Chapter 4** describes quantitative and functional antibody responses to the vaccination scheme. Immunophenotyping of lymphocyte subsets during the first 15 months following transplantation is described in **chapter 5**. Reconstitution of T-cell function, measured by determination of cytokine production and T-cell proliferation, is described in **chapter 6**.
Finally, **chapter 7** comprises a summary and general discussion of the findings of this thesis.

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CHAPTER 2

Influenza virus vaccination and booster in B-cell chronic lymphocytic leukaemia patients

Influenza virus vaccination and booster in B-cell chronic lymphocytic leukaemia patients

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Summary

Influenza vaccination is recommended in patients with B-cell CLL. Because response rates are often low, it is of interest to evaluate antibody response to single and booster vaccination with influenza A and B virus vaccine in these patients. Twenty patients with B-CLL received two subunit virus vaccine injections with a 21-day interval. Antibody titres were determined before and 21 days after single and booster vaccination. The serologic response was expressed using the following criteria: 1) response rate, i.e. the proportion of subjects with at least a four-fold titre increase; 2) the protection rate, i.e. the proportion of subjects exceeding the threshold of 100 (influenza A) or 200 (influenza B); 3) the mean fold increase (MFI), i.e. the difference between the logarithmated geometric mean titres of pre- and post-vaccination sera. Response rates were 5% for influenza A and 15% for B after single and 15% for A and 30% for B after booster vaccination. Protection rates were 0% for influenza A and 25% for B after single vaccination. Protection rates were 5% (H1N1) and 10% (H3N2) for influenza A and 30% for B after booster. The MFI ± SD (range) after booster vaccination was 0.26 ± 0.33 (0-1.00), 0.17 ± 0.34 (0-1.00) and 0.35 ± 0.34 (0-1.20) for H1N1, H3N2 and influenza B, respectively. In conclusion, in this study with B-CLL patients, immune response to influenza vaccination was poor. Single and booster vaccinations with influenza virus vaccine are not of great value in patients with B-cell CLL.

Introduction

Chronic lymphocytic leukaemia (CLL) is a frequent disease and accounts for over 25% of leukaemia seen in clinical practice[1]. The disease mainly affects the elderly and is associated with a relatively high life expectancy. In more than 90% of the cases, the cells are a monoclonal population of B lymphocytes[2].
CLL-patients are prone to infectious complications with high morbidity and mortality. In elderly and immunocompromised people, influenza vaccination is recommended[3]. Although influenza vaccination is indicated in CLL patients, the response rate is often low. Grant Stiver *et al* studied antibody response to influenza A and B vaccination in cancer patients[4]. A four-fold or greater increase in antibody titre was found in 36% and 32% for influenza A and B, respectively. Four patients with CLL who were receiving chemotherapy at the time of vaccination were included. A four-fold increase did not occur in any of these patients. Gross *et al* also suggest that antibody response to influenza virus is significantly weaker in patients receiving chemotherapy[5].
A booster vaccination might result in higher response rates although previous reports show conflicting results. Rautenberg *et al* did not find a significant increase in response or protection rate after booster vaccination in haemodialysis patients[6]. Bouter *et al* studied influenza vaccination with booster in patients with type 1 diabetes mellitus. Booster vaccination increased the protection rate for the influenza B strain only[7]. Gross *et al* found no enhanced immune response after booster vaccination in the elderly[8].
The main cause of immunoincompetence in patients with B-CLL is a decreased capacity to produce immunoglobulins[9]. Because low response rates to a single influenza vaccination are to be expected, it is of interest to study whether the immune response can be improved after a booster vaccination, as has been suggested previously in patients with other haematological malignancies[10].

Material and methods

Patients

In 1997, twenty patients with immunophenotypically proven B-CLL who visited our outpatient clinic, were included in this study. Clinical characteristics are given in *Table* 1. Seven patients had been treated previously with chemotherapy but no

patient had received chemotherapy during the three months prior to vaccination. Data concerning prior vaccination with influenza virus vaccine were obtained by interview.
All patients received an intramuscular injection of 0.5 ml commercially available inactivated subunit virus vaccine (Influvac®, Duphar, Amsterdam, the Netherlands) on day 0. The 1997-vaccine contained 15 µg haemagglutinin (HA) of A/Bayern/7/95 (H1N1) virus, 15 µg HA of A/Wuhan/359/95 (H3N2) virus and 15 µg HA of B/Beying/184/93 virus.
A booster vaccination with the same vaccine was given 3 weeks later. Patients were monitored for fifteen minutes after injection for any immediate reaction. All patients gave informed consent for the study and the use of their data.

Methods
Blood samples were taken on day 0, 21 and 42. Whole blood count including leukocyte differentiation, gammaglobulin-fraction, total levels of IgA, IgM and IgG and soluble CD23-levels were determined. Hypogammaglobulinaemia was defined as a gammaglobulin level < 6.2 g/L. Normal ranges of serum immunoglobulins were defined as IgA, 0.5 to 4.0 g/L; IgM, 0.4 to 2.3 g/L; and IgG, 7.0 to 15.0 g/L. In one patient, determination of gammaglobulin- and total IgG-levels was unreliable because of a paraproteinaemia of IgG-kappa. Soluble CD23-concentration, which is considered a suitable marker for disease progression, was measured using Medgenix EASIATM assay (Biosource, Fleurers, Belgium). Blood samples for determination of pre-vaccination and post-vaccination antibody titres against influenza virus were separated immediately after blood collection and clotting and stored at −20°C until titration. Influenza strains were propagated in embryonated hen's eggs. Infectious egg fluids of this strain were treated with aether and the watery phase was used in the serologic tests.
Serum haemagglutination inhibition (HI) titres were determined twice by standard methods simultaneously in pre- and post-vaccination sera. Titres were expressed as reciprocals of the dilution showing 50% HI with three haemagglutination units of the antigen. The geometric means of the two determinations were used for further calculations. Negative titres (<5) were arbitrarily regarded as 2. With the method used, an HI titre of 100 is thought to be protective against infection with influenza A. No protection threshold is known for aether-treated influenza B strains. In this study, an HI titre of 200 was assumed to be protective. The serologic response upon vaccination was expressed using the following criteria:

1) the response rate, i.e. the proportion of subjects with a 4-fold or greater titre increase after vaccination; 2) the protection rate i.e. the proportion of subjects exceeding the threshold titre of 100 or 200 after vaccination; 3) the mean fold increase (MFI), i.e. the difference between the logarithmated geometric mean titres of pre-vaccination sera and sera after booster vaccination.

Statistical analysis
Statistical analyses were performed by using Student t-test, chi-square test and Pearson's correlation test as appropriate. The Excel 97 package was used. P values < 0.05 were considered statistically significant. Data are given as mean ± standard deviation (range).

Results

In three patients, gammaglobulin levels were less than 6.2 g/L. Mean level of gammaglobulin in all patients was 8.8±2.6 g/L (4.4-12.8). Mean levels of IgM, IgG and IgA were 0.31±0.17 (0.08-0.6), 7.16±2.48 (2.3-11.9) and 0.95±0.91 g/L (0.10-4.1), respectively (*Table* 1). Mean level of sCD23 was 158.6±126.1 g/L (15-416). Fifteen patients received prior vaccination with influenza virus vaccine.

Table 1. Clinical characteristics of patients

Patients, no	20
Mean age, yrs ± SD (range)	71.4 ± 7.3 (52-85)
Male:female, no	13:7
Duration of disease, yrs ± SD (range)	5.1 ± 5.0 (1-21)
Stage of disease according to Rai, no (%)	Rai-0: 4 (20)
	Rai-1: 2 (10)
	Rai-2: 6 (30)
	Rai-3: 5 (25)
	Rai-4: 3 (15)
Previously chemotherapy, no (%)	7 (35)

After a single vaccination, the response rate was 5%, 5% and 15% for H1N1, H3N2 and B, respectively. After booster vaccination, the response rate was 15%, 15% and 30% (*Table* 2). Two patients (10%) responded to all vaccine subunits.

Response rates did not increase significantly after single or booster vaccination.

There was no correlation between responders and age, duration of disease and levels of gammaglobulin, IgM, IgG, IgA or sCD23.

Table 2. Response[#] and protection[§] rates after single and booster vaccination for influenza A (H1N1 and H3N2) and influenza B.

	H1N1	H3N2	B
	n=20	n=20	n=20
No. of responders (%) after single vaccination	1 (5)	1 (5)	3 (15)
No. of responders (%) after booster vaccination	3 (15)	3 (15)	6 (30)
No. of patients (%) protected before vaccination	0	1 (5)	3 (15)
No. of patients (%) protected after single vaccination	0	0	5 (25)
No. of patients (%) protected after booster vaccination	1 (5)	2 (10)	6 (30)

[#]*response rate: the proportion of subjects with a four-fold or greater titre increase*

[§] *protection rate: the proportion of subjects exceeding the threshold of 100 (influenza A) or 200 (influenza B)*

The protection rate was 0% for H1N1 and H3N2 and 25% for B after a single vaccination. Of 5 patients who had protective antibody titres after a single vaccination for influenza B, 3 already had protective antibody titres before vaccination. These 3 patients all had an increase in antibody titre against influenza B after single vaccination (data not shown). After booster vaccination, protection rates were 5%, 10% and 30% for H1N1, H3N2 and B, respectively (*Table 2*). Protection rates did not increase significantly after single or booster vaccination. For H1N1 and H3N2, there was no correlation between responders and age, duration of disease and levels of gammaglobulin, IgM, IgG, IgA or sCD23. For influenza B, all patients with pre- or post-vaccination protective antibody titres, had significantly higher concentrations of gammaglobulin ($p<0.002$) and IgG ($p<0.004$). Other factors showed no correlation with protective antibody titres.
The mean fold increase (mean ± SD, range) after booster vaccination was 0.26 ± 0.33 (0-1.00); 0.17 ± 0.34 (0-1.00); and 0.35 ± 0.34 (0-1.20) for H1N1, H3N2 and influenza B, respectively (*Figure 1*). For influenza A and B, the MFI was not statistically significant after single or booster vaccination. There was no correlation between MFI and levels of IgG, IgA, IgM or sCD23 (data not shown).

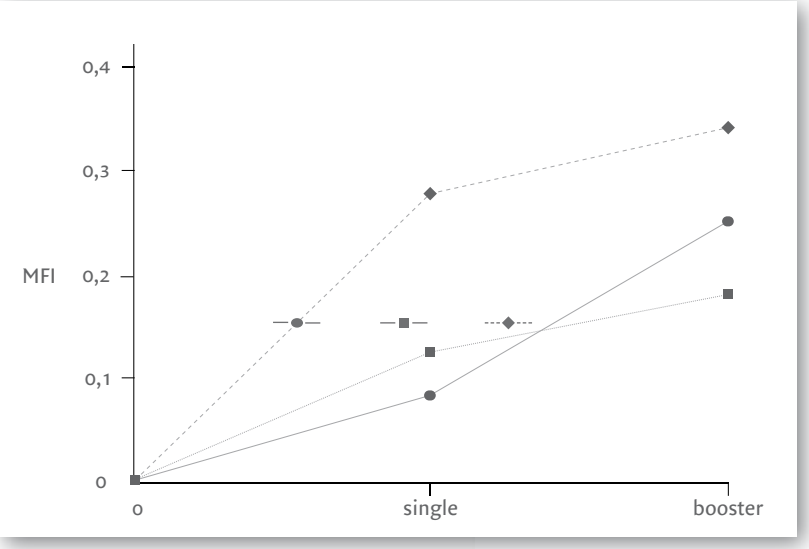

Figure 1: Mean fold increase (i.e. the difference between the logarithmated geometric mean antibody titres) after single and booster vaccination . H1N1, H3N2, B.

Discussion

Patients with B-CLL often have a diminished immune response to infections and are prone to serious influenza infections with high morbidity and mortality[9]. In Europe, similar as has been recommended by the World Health Organisation[3], vaccination with influenza virus vaccine has been advised for these patients. Previous studies have shown varying responses to influenza vaccination in cancer patients[12,10,13,4]. Although Brydak *et al* found significant MFI's in a study with patients receiving chemotherapy[14], other studies showed low responses after influenza vaccination during chemotherapy[5,15]. Gribabis *et al* found an initially satisfactory response to influenza vaccination in B-CLL patients but a significant decline in antibody levels on days 30 and 60[12]. This observation led to the suggestion that a booster vaccination is necessary for maintenance of protective antibody levels, as has been demonstrated for hepatitis B vaccine in dialysis patients[16]. Booster vaccination with influenza virus vaccine showed, however, a limited improvement in antibody response in patients with diabetes mellitus and end stage renal disease[7,6] and no enhanced immune response after booster vaccination was observed in elderly people[8]. A remarkable observation that annually repeated vac-

cination might successively decrease protection was published by Hoskins *et al*[17]. However, this study showed shortcomings in study design and the results could not be reproduced by others[18,19,20]. The Hoskins' paradox should, therefore, not be used to discourage multiple vaccination.

In this study, antibody response to single and booster influenza vaccination was studied in B-CLL patients. The study was limited with respect to study size and lack of healthy controls. Furthermore, antibody titres and not clinical efficacy of influenza vaccination, was measured. The response rate (i.e. a four fold or more increase in antibody titres) after single vaccination in this study was 5% and 15% for influenza A and B, respectively. This is very poor compared to other studies. In patients with Graves' disease, response rates after single vaccination were over 70%[21]. In cancer patients, response rates were 36% and 32% for influenza A and B, respectively[4]. In this study with B-CLL patients, response rates after booster vaccination were 15% and 30% for influenza A and B, respectively. Protection rates (i.e. antibody levels in the protective range) after single vaccination were 0% for influenza A and 25% for influenza B. After booster vaccination, protection rates were 5% (H1N1) and 10% (H3N2) for influenza A and 30% for influenza B. This marginally improvement in protection and response rates after booster vaccination is consistent with studies in patients with diabetes or end stage renal disease[7,6].

The mean fold increase (MFI) was also calculated in this study. The MFI was disappointingly low for all vaccine components (0.26 for H1N1, 0.17 for H3N2 and 0.35 for influenza B). Other studies reported MFI's of 1.00 – 2.10 in controls and patients with type 1 diabetes mellitus or Graves' disease[7,22,21].

Hypogammaglobulinaemia is frequently seen in CLL patients and is associated with decreased immune responses[23]. However, in our study, only 15% of the patients showed a hypogammaglobulinaemia. Although age may affect the degree of response to influenza vaccine[24], some studies have not been able to correlate diminished responsiveness to influenza vaccine with age[20]. The mean age in our study was 71 years but responders were not statistically younger than non-responders (data not shown). Thus, other factors must contribute to the low antibody response found. The immunologic response to booster influenza vaccination is a T-cell dependent reaction. Deterioration of T-cell function has been observed in B-CLL[25]. This might be an explanation for the lack of a booster effect in CLL patients after multiple vaccinations with influenza virus vaccine. Furthermore, B-CLL patients have a monoclonal malignant proliferation of B cells.

These lymphocytes have a specific rearrangement and produce only one type of antibody[2]. It is also postulated that non-malignant B cells in B-CLL patients are downregulated by the microenvironment induced by the malignancy[26]. Malignant B cells can produce tumour growth factor-β. Hyporesponsiveness of both malignant and non-malignant lymphocytes may be partly caused by increased levels of such cytokines. Therefore, even with normal gammaglobulin levels prior to vaccination, antibody production in response to vaccination might be insufficient[9].
New anti-viral drugs such as zanamivir and oseltamivir will become important in preventing influenza infections in people at risk. These drugs inhibit the neuraminidase enzyme that is essential for viral propagation. Zanamivir has been shown to prevent influenza infection in 67% of a group consisting of healthy adults[27]. Further research is needed to establish the role of these anti-virals in immunocompromised patients.
In summary, antibody response to influenza A and B vaccination was very low in B-CLL patients. Booster vaccination showed limited improvement in response and protection rates. Although single vaccination with influenza virus vaccine is common practice in patients with B-cell CLL, no significant improvement in protection rate after single and booster vaccination can be found. Both single and booster vaccination with influenza virus vaccine is not of great value in patients with B-cell CLL.

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CHAPTER 3

The effect of ranitidine on antibody responses to polysaccharide vaccines in patients with B-cell chronic lymphocytic leukaemia

The effect of ranitidine on antibody responses to polysaccharide vaccines in patients with B-cell chronic lymphocytic leukaemia

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Summary

The effects of ranitidine treatment on vaccination-induced antibody responses in patients with chronic lymphocytic leukaemia (CLL) were analysed. 50 CLL patients were vaccinated with tetanus conjugated *Hib* vaccine and a 23-valent pneumococcal polysaccharide vaccine with (n=25) or without (n=25) ranitidine treatment in a matched case-control setting. Anti tetanus toxoid (TT), anti-*Hib* and anti-pneumococcal antibody levels were determined before and after vaccination. Additionally, cytokine levels were assessed in patients treated with ranitidine. Vaccination-induced increases in anti-*Hib* and anti-TT antibody levels were higher in the ranitidine group compared to the control group. Anti-pneumococcal antibody responses were not improved by administration of ranitidine. Higher levels of IL-18 were found in patients treated with ranitidine compared to healthy controls. Levels of IL-6, IL-8, IL-18, RANTES, IP-10, sVCAM-1 and sICAM-1 were within normal ranges and did not change during ranitidine treatment. Ranitidine treatment improves vaccination-induced T-cell dependent antibody responses in patients with CLL but has no beneficial effect on the response to vaccination with unconjugated polysaccharide antigens.

Introduction

Chronic lymphocytic leukaemia (CLL) is the most common type of leukaemia and affects mainly elderly. CLL is a clonal proliferation of lymphocytes, mostly of B-cell lineage[1]. Depending on stage and cytogenetics, prognosis is often good with a mean life expectancy of about 150 months in Rai stage 0. However, infections are common and often lead to serious complications[2,3]. Encapsulated bacteria such as *Haemophilus influenzae* type b *(Hib)* and *Streptococcus pneumoniae* are major pathogens. Therefore, vaccination with *Hib* conjugate vaccine and pneumococcal polysaccharide vaccine is recommended in patients with CLL and other haematological malignancies[4]. In most other groups at risk, these vaccines have been demonstrated to be immunogenic[5,6]. However, CLL patients show disappointing low responses to vaccination with *Hib* conjugate and pneumococcal vaccine[7,8]. Mellemgaard *et al*[9] observed that the antibody response to tetanus toxoid (TT), a T-cell dependent vaccine, was significantly better during treatment of CLL patients with the gastric-acid suppressive drug ranitidine. In that same study, the antibody response to the T-cell independent pneumococcal vaccine did not improve. In a more recent study, this group also observed a higher response rate to *Hib* conjugate vaccine during ranitidine treatment[10]. The positive effects of ranitidine on the antibody response to vaccination might be due to interference of ranitidine with the action of histamine on histamine type 2 receptors. Histamine regulates both humoral and cellular immune responses[11]. Histamine can inhibit immunoglobulin production, down regulate lymphocyte proliferation and has an effect on the production of various cytokines. Activation of histamine receptor type 2 can have mainly negative effects on immune regulation[11]. Ranitidine is a histamine type 2 receptor antagonist, thereby blocking the effects of histamine.
Despite earlier studies indicating a positive effect of ranitidine treatment on antibody responses in patients with CLL[9,10], ranitidine treatment during vaccination has not become common practice. However, enhancement of antibody responses to vaccination in this group of patients with high risk of infections, is a highly attractive method of infection prevention. Therefore, further evaluation of the effects of ranitidine treatment on antibody responses is worthwhile. We conducted a matched case-control trial to compare antibody responses to pneumococcal polysaccharide vaccination and to *Hib* conjugate in CLL patients with and without ranitidine treatment. Furthermore, we analysed the influence of ranitidine on cytokine levels.

Materials and methods

Patients and study design

Fifty patients with immunophenotypically proven B-CLL were studied in a matched case-control setting. In a previous study, hypogammaglobulinaemia and disease stage were correlated with antibody responses to pneumococcal and *Hib* vaccination[8]. Therefore, in the current study, patients were matched for disease stage according to Rai[12] and hypogammaglobulinaemia. Exclusion criteria were chemotherapy during the last two months, intermittent gammaglobulin therapy and treatment with histamine receptor antagonists before study entry. The study was performed in three teaching hospitals in the Netherlands. Identical inclusion criteria as well as CLL-treatment protocols were used at each study location. Case patients (n=25) were prescribed ranitidine (300 mg once daily, Zantac-effervescent granules; Glaxo, Copenhagen, Denmark) for the whole study period of 49 days. Control patients (n=25) received no ranitidine. Four weeks after study entry, all patients (n=50) received an intramuscular injection of PRP-TT (Act-Hib®; Pasteur-Merieux Laboratories, Lyon, France) that contains 10 µg of *Haemophilus influenzae* type b polysaccharide covalently linked to tetanus toxoid (± 24 µg). At the same time, patients were given an injection of a 23-valent pneumococcal polysaccharide vaccine (Pneumovax-23®; MSD, Rahway, NJ) containing 25µg of purified capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. After the injections, patients were monitored for 15 minutes for any immediate reactions. Patients were asked to note any local or systemic reactions during 3 days after vaccination in a structured questionnaire. The study was approved by the Regional Ethical Committee. All patients gave written informed consent before study entry.

Detection of antibodies by Enzyme-linked Immunosorbent Assay (ELISA) and assessment of cytokine levels

A complete blood cell count including leukocyte differentiation, total levels of serum IgM, IgG, IgA, IgG subclasses and gammaglobulin-fraction was determined in blood samples obtained at study entry. Normal ranges of serum immunoglobulins were defined as IgA, 0.5-4.0 g/l; IgM, 0.4-2.3 g/l; and IgG, 7.0-15.0

g/l. Hypogammaglobulinaemia was defined as a gammaglobulin level ≤ 6.2 g/l.
In patients who were receiving ranitidine treatment, serum samples for determination of antibodies against *Hib*, *Streptococcus pneumoniae* and TT were obtained at study entry (day 0), after 4 weeks of ranitidine treatment when the vaccines were given (day 28) and 3 weeks after vaccination (day 49). Antibody levels after 4 weeks of ranitidine treatment were considered as baseline antibody titres and were compared to baseline antibody levels in the control group. In control patients, serum samples for antibody measurements were obtained before vaccination (day 28) and 3 weeks after vaccination (day 49).
Pre- and post-immunization serum samples were pre-incubated with excess (50 µg/ml) pneumococcal common cell wall polysaccharide (CPS) overnight at 4°C before analysis to block anti-CPS antibodies[13]. Next, serial dilutions of serum samples were incubated on ELISA plates coated with 10 µg/ml pneumococcal polysaccharide serotype 3, serotype 4 or serotype 9V. The IgG anti-*Hib* and anti-TT antibody titres were determined by ELISA in wells coated with 1 µg/ml *Haemophilus influenzae* type b polysaccharide conjugated to human serum albumin (HbO-HA Lot 17, Wyeth Lederle Vaccines, NISCB, UK) or TT (5Lf/ml in 0.05 M carbonate buffer pH 9.6), respectively[14].
All antibody titres were log-transformed and geometric mean titres (GMT) calculated. For statistical analysis, antibody titres that were less than the limit of the assay's sensitivity were assigned values equal to one-half of the lower detection limit. Data are also reported in terms of proportions of patients who showed seroconversion (defined as a ≥ 4-fold increase in antibody titre) and who attained protective antibody titres. For *Hib*, anti-PRP antibody concentrations ≥ 1.0 µg/mL are considered to correlate with long-term protection[15], whereas for tetanus, antibody concentrations of 0.1 IU/mL are correlated with long-term protection[16]. For *Streptococcus pneumoniae*, anti-pneumococcal antibody titres of ≥0.35 µg/mL have been recommended by the WHO as an estimate of the threshold concentration for protection against invasive disease[17].
In patients who were treated with ranitidine, cytokines were determined in sera obtained at day 0 (before start of treatment with ranitidine) and at day 28 (after four weeks of treatment, before vaccinations were given). The cytokines IL-6 and IL-18, chemokines IL-8 (CXCL8), IP-10 (XCL10) and RANTES (CCL5), and soluble adhesion molecules sVCAM-1 and sICAM-1 were determined by multiplex immunoassay as described by De Jager *et al*[18].

Statistics
Comparisons of GMT before and after vaccination (paired samples) were performed using the Wilcoxon signed rank test, comparisons between 2 groups (unpaired samples) were performed using the Mann-Whitney U test. For categorical variables, univariate, between groups, comparison was performed by means of the Chi-square test.

Results

A total of 50 patients were included. Baseline characteristics are given in *Table 1*. No significant differences were noted in sex distribution, age, duration of disease, therapy or baseline immunological parameters between the ranitidine and control group. No serious adverse reactions after vaccination were reported. Treatment with ranitidine during the 28 days before vaccination as such did not induce changes in *Hib* or pneumococcal antibody titres (data not shown).
In *Table 2*, GMT, protection rates and seroconversion rates for *Hib* vaccination are given. Vaccination resulted in a significant increase in anti-*Hib* antibodies in the ranitidine group and in controls. When comparing post-vaccination antibody titres, a significant higher anti-*Hib* antibody titre was found in the ranitidine group than in the control group. Since the level of pre-vaccination antibodies might have impact on the magnitude of response, we also compared the increases in antibody levels between both groups and found a significant higher increase in anti-*Hib* antibody levels in the ranitidine group compared to the control group. The number of patients with anti-*Hib* antibody levels in the protective range was significantly higher in the ranitidine group than in the control group. Seroconversion rates were not statistically significant different in both groups.

Table 1. Baseline characteristics

		Ranitidine (n=25)	Controls (n=25)
Male, n (%)		16 (64)	17 (68)
Age, years (± SD)		68.4 (± 11.8)	70.0 (± 9.0)
Rai, n (%)	0	14 (56)	14 (56)
	1	5 (20)	5 (20)
	2	6 (24)	6 (24)
Disease duration, months (± SD)		41 (± 44)	33 (± 39)
Previous chemotherapy, n (%)		5 (20)	4 (16)
Leukocytes, x109 g/l (± SD)		49 (± 55)	41 (± 39)
IgG, g/l (± SD)		8.7 (± 3.6)	8.2 (± 2.1)
IgA, g/l (± SD)		1.3 (± 0.7)	1.3 (± 0.9)
IgM, g/l (± SD)		0.6 (± 0.4)	0.5 (± 0.3)
Gammaglobulin, g/l (± SD)		10.5 (± 4.0)	9.9 (± 2.8)
Hypogammaglobulinaemia, no (%)		3 (12)	3 (12)
IgG1, g/l (± SD)		6.9 (± 3.1)	5.7 (± 2.0)
IgG2, g/l (± SD)		2.6 (± 0.9)	2.6 (± 1.1)
IgG3, g/l (± SD)		0.5 (± 0.3)	0.4 (± 0.2)
IgG4, g/l (± SD)		0.3 (± 0.3)	0.3 (± 0.2)

Table 2. *Haemophilus influenzae* type b: geometric mean titre (GMT), protection rate and seroconversion rate

	ranitidine		controls	
	pre	post	pre	post
GMT	3.18	15.00[a,b]	1.93	4.07[a,b]
protection, no. (%)	21 (84%)[c]	23 (96%)[d]	14 (56%)[c]	15 (60%)[d]
seroconversion, no. (%)		13 (54%)		7 (28%)

Note: paired pre- and post-vaccination antibody titres were available for 24 patients in the ranitidine group and 25 patients in the control group.

[a] $p < 0.035$ for pre- versus post-vaccination

[b] $p = 0.046$ for ranitidine versus controls

[c] $p = 0.031$ for ranitidine versus controls

[d] $p = 0.003$ for ranitidine versus controls

Anti-TT antibody levels increased in both groups *(Table 3)*. A significant lower protection rate before vaccination in the ranitidine group was accompanied by a significant higher increase in anti-TT antibody titres and seroconversion rate compared to controls. However, post-vaccination anti-TT antibody levels did not differ between the groups *(Table 3)*.

Table 3. Tetanus toxoid (TT): geometric mean titre (GMT), protection rate and seroconversion rate.

	ranitidine		controls	
	pre	post	pre	post
GMT	0.12	1.03[a]	0.24	0.46[a]
protection, no. (%)	8 (42%)[b]	14 (74%)	18 (86%)[b]	18 (86%)
seroconversion, no. (%)		13 (68%)[c]		5 (24%)[c]

Note: TT antibody titres were determined in 19 patients of the ranitidine group and in 21 patients of the control group.

[a] $p < 0.05$ for pre- versus post-vaccination

[b] $p = 0.004$ for ranitidine versus controls

[c] $p = 0.005$ for ranitidine versus controls

In *Table* 4, GMT, protection rates and seroconversion rate for anti-pneumococcal antibodies are given. Overall, no significant differences between both groups were noted.

Table 4. Pneumococcal polysaccharide: geometric mean titre (GMT), protection rate and seroconversion rate.

	ranitidine		controls	
	pre	post	pre	post
GMT PS3	0.18	0.23[a]	0.17	0.19
GMT PS4	0.70	0.72	0.35	0.55
GMT PS9	0.37	0.49	0.35	0.59[b]
protection, no. (%)	12 (48%)	15 (63%)	10 (40%)	14 (56%)
seroconversion, no. (%)		none		3 (12%)

Note: paired pre- and post-vaccination antibody titres were available for 24 patients in the ranitidine group and 25 patients in the control group. PS3, pneumococcal serotype 3; PS4 pneumococcal serotype 4; PS9, pneumococcal serotype 9.

[a] $p < 0.001$ for pre- versus post-vaccination

[b] $p = 0.033$ for pre- versus post-vaccination

Histamine receptor antagonists have been described to influence the in vitro secretion of IL-6, IL-8 and IL-18 [19,20]. Therefore, a potential mechanism by which ranitidine could exert its immunomodulating effects, is through alteration of serum cytokine and chemokine levels. Serum levels of IL-6, IL-8, IL-18, RANTES, IP-10, sVCAM-1 and sICAM-1 were determined in 19 patients of the ranitidine group. Higher levels of IL-18 were found in these patients compared to healthy controls (mean IL-18 level 382 pg/ml in patients versus 6 pg/ml in controls, p-value < 0.001). Levels of IL-6, IL-8, IL-18, RANTES, IP-10, sVCAM-1 and sICAM-1 all were within normal ranges and did not change during ranitidine treatment (data not shown).

Discussion

In the current study, patients with B-CLL were vaccinated with TT-conjugated *Hib* vaccine and pneumococcal vaccine. Patients who were treated with ranitidine had higher vaccination-induced increases in anti-*Hib* and anti-TT antibody levels

compared to controls. Ranitidine treatment had no effect on anti-pneumococcal antibody responses.

Histamine has diverse, but ill-understood effects on cells of the immune system. Binding of histamine to histamine receptors on monocytes, neutrophils, dendritic cells and lymphocytes can have either positive or negative effects[11, 21-23]. Part of these variable results may be due to the existence of different types (type 1, 2, 3, and 4) of membrane histamine receptors[24-26]. Binding of histamine to histamine receptor type 2 on type 2 T-helper (Th2) cells, results in cyclic-AMP formation[27, 28]. Cyclic-AMP downregulates T-cell proliferation and IL-4 and IL-13 production and thus stimulation of proliferation and differentiation of B-cells. Moreover, direct modulation of B-cell activation and immunoglobulin production by histamine, as well as differentiation of dendritic cells, has been described[11,23]. Ranitidine has been shown to completely abrogate the formation of cAMP by Th2 cells[23]. Because of these immunomodulatory properties of ranitidine, we were interested whether treatment of CLL patients with ranitidine would result in an alteration of plasma cytokine patterns. We therefore analysed IL-6, IL-8, IL-18, IP-10, RANTES, sICAM-1 and sVCAM-1 and found higher serum levels of IL-18 in the CLL patients as compared to healthy controls. Dysregulated expression of IL-18 and IL-18 mRNA in vitro has been described in chronic B-cell lymphoproliferative disorders and might function as a means to escape the host's immune system[29]. Treatment with ranitidine had no effect, neither positive nor negative, on serum IL-18 levels, nor on the levels of the other cytokines and chemokines measured.

For many years, defects in humoral immunity, reflected in hypogammaglobulinaemia, have been considered the major factor causing immune deficiency in CLL[30]. However, deficient T helper cell function has also been described in patients with CLL[31,32]. In the current study, patients with B-CLL were vaccinated with TT-conjugated *Hib* vaccine and pneumococcal vaccine. Vaccination with polysaccharide antigens such as the pneumococcal vaccine, elicits a T-cell independent type-2 antibody response that lacks immunological memory. In *Hib*-conjugate vaccine, the carrier protein tetanus toxoid is coupled to the polysaccharide. The conjugate is expected to elicit a T-cell dependent anti-polysaccharide antibody response with induction of immunological memory.

Mellemgaard *et al* showed increased antibody responses to TT in a group of 23 patients with B-CLL who received ranitidine compared to non-treated B-CLL patients[9]. In that same study, no benefit of ranitidine was found for the response

to pneumococcal vaccination. The authors state that ranitidine only improves T-cell dependent antibody responses such as to the *Haemophilus influenzae* conjugate vaccine. Indeed, Jurlander *et al* found an improved antibody response to conjugated *Haemophilus influenzae* vaccine in patients with B-CLL who were treated with ranitidine[10].

In the current study on 50 patients with CLL, significant higher increases in anti-*Hib* and anti-TT antibody levels were found in patients treated with ranitidine compared to controls. Additionally, the TT seroconversion rate was higher in the ranitidine group than in controls. Before vaccination, 42% of patients in the ranitidine group had anti-TT antibody levels in the protective range, compared to 86% in the control group. A low pre-vaccination anti-TT antibody titre might attribute to the higher increase in anti-TT antibody levels and the higher seroconversion rate in the ranitidine group following vaccination. For anti-*Hib* antibody responses, however, this does not hold true. Despite the finding that more patients in the ranitidine group had pre-vaccination anti-*Hib* antibody levels in the protective range compared to the control group, a significant higher increase in anti-*Hib* antibody levels was found in the ranitidine group. For anti-pneumococcal antibody responses, no effect of ranitidine was found. These findings suggest a beneficial effect of ranitidine treatment on T-cell dependent antibody responses to vaccination.

One of the differences between the current study and the earlier studies by Mellemgaard *et al* and Jurlander *et al* is the dosage of ranitidine. Although we used a lower dosage compared to former studies (300 mg once daily compared to 300 mg twice daily), we could confirm the beneficial effects of ranitidine on vaccination-induced anti-*Hib* and anti-TT antibody levels. Secondly, the interval between vaccination and antibody evaluation differs between former studies (28 and 45 days) and ours. Since antibody evaluation is generally performed 3-4 weeks after vaccination[7, 8, 13], we used a 21 days interval between vaccination and antibody analysis.

In conclusion, the results of this study suggest that T-cell dependent antibody responses to vaccination in patients with CLL can be improved by administration of the histamine type 2 receptor antagonist ranitidine.

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CHAPTER 4

Development of functional *Haemophilus influenzae* type b antibodies after vaccination of autologous stem cell transplant recipients

Development of functional *Haemophilus influenzae* type b antibodies after vaccination of autologous stem cell transplant recipients

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Summary

The conditioning regiments for autologous stem cell transplantation (aSCT) can lead to impairment of the immune system and susceptibility to infections. Although infectious complications are more prominent in allogeneic transplantation, it is also a major source of morbidity in patients undergoing aSCT. Therefore, vaccination of patients after stem cell transplantation has been recommended. We analysed quantitative and qualitative aspects of the antibody response after *Haemophilus influenzae* type b (*Hib*)-conjugate vaccination in autologous stem cell transplant recipients. Sixteen patients with multiple myeloma (n=13) and non-Hodgkin's lymphoma (n=3) received a conjugated *Hib* vaccine at 6, 8 and 14 months after transplantation. Antibody titres were determined by ELISA. Functionality of anti-*Hib* antibodies was assessed by measurement of antibody avidity and opsonophagocytosis. Adequate anti-*Hib* antibody responses (i.e. a four fold or greater increase in antibody levels in addition to a minimal titre of 50 U/ml corresponding to 18.8 microgram/ml) were achieved after two vaccinations in 75% (12 of 16) of the patients, and after the third vaccination in 93% (13 of 14) of the patients. Repeated vaccination induced maturation of antibody avidity as demonstrated by increased resistance to NaSCN treatment. Anti-*Hib* antibodies supported phagocytosis by polymorphonuclear cells after multiple vaccinations. Our data show that multiple vaccinations with *Hib*-conjugate vaccine in autologous stem cell recipients result in high antibody response rates and functional maturation of antibodies.

Introduction

Autologous stem cell transplantation (aSCT) has become a common procedure in the treatment of patients with haematological malignancies such as multiple myeloma and non-Hodgkin's disease. The conditioning regiment for aSCT leads to impairment of the immune system and patients are susceptible to infections during the recovery phase[1]. Although infectious complications are more frequently seen in allogeneic transplantation, it is also a major source of morbidity in patients undergoing autologous stem cell transplantation[2,3]. Post-transplantation bacterial pneumonia is often caused by encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (*Hib*)[2,4,5]. Vaccination of stem cell transplant recipients with pneumococcal vaccine and *Hib* therefore has been recommended by health institutions such as the Centers for Disease Control and Prevention, the American Society of Hematology and the European Group for Blood and Marrow Transplantation[6,7,8]. Several studies have described the antibody responses to vaccination with *Hib* in autologous and allogeneic transplant recipients[9,10,11]. In most studies, adequate antibody responses were found after multiple vaccinations with *Hib*-conjugate vaccine. A response to vaccination is often quantitatively expressed as antibody titres, and the antibody titre as determined by ELISA is often considered a surrogate marker of clinical effectiveness of vaccination. Determination of avidity and phagocytosis-inducing capacity of antibodies can, however, provide important information regarding the functional activity of antibodies[12,13,14]. Therefore, we conducted a prospective follow-up study to determine quantitative and qualitative aspects of the humoral immune response to multiple vaccinations with conjugated *H. influenzae* type b vaccine in autologous stem cell recipients.

Design and methods

Patients and vaccination schedule

Sixteen adult patients with non-Hodgkin's lymphoma (n=3) or multiple myeloma (n=13), who underwent autologous stem cell transplantation (aSCT), were included. Exclusion criteria were vaccination with *Hib* vaccine in the first three months after transplantation and recurrence of tumour or start of chemotherapy

within six months after aSCT. Patients with multiple myeloma or amyloidosis received high dose melphalan (cumulative dose 200 mg/m^2), whereas patients with NHL received the BEAM-regimen (cumulative doses: BCNU 300 mg/m^2, ARA-C 800 mg/m^2, VP16 800 mg/m^2, melphalan 140 mg/m^2) as conditioning therapy. All patients gave written informed consent. The study was approved by the local Ethical Committee.
Six months after aSCT, patients were vaccinated with *Hib* (PRP-T vaccine, polyribosylribitolphosphate (PRP) conjugated to tetanus toxoid, Pasteur Mérieux Sérums et Vaccins, Lyon, France). Patients received a booster vaccination 2 and 8 months later, i.e. 8 and 14 months after transplantation. All vaccines were administered intramuscularly in the upper arms. Patients were observed fifteen minutes after each vaccination and were asked to record local and systemic reactions on a case report form during three days after vaccination.

Antibody levels
IgG antibody levels to *H. influenzae* were measured by ELISA as described previously[15,16]. Serum samples were taken before vaccination and 3 weeks after each vaccination. Pre- and post-vaccination serum samples from individual patients were analysed on the same ELISA plate. The anti-*Hib* antibody titres were determined by ELISA in wells coated with 1.0 microgram/ml *H. influenzae* type b polysaccharide conjugated to human serum albumin (HbO-HA Lot 17, Wyeth Lederle Vaccines, NISCB, UK). After washing, alkaline phosphatase-conjugated goat anti-human IgG (Camarillo, CA) was incubated for 2 hours at 37°C. Following washing, the reaction was developed with substrate (*p*-Nitrophenyl Phosphate, Sigma, St. Louis, MO) during 20-45 minutes at room temperature after which the reaction was stopped with 2.4 M NaOH . Results were read with a plate reader (Milenia Kinetic Analyzer, Diagnostic Products Corporation, Los Angeles, CA) at 405 nm. Anti-*Hib* IgG antibodies were expressed in micrograms/ml, relative to standard serum (human *Haemophilus* reference serum, lot 1983, kindly provided by Dr. C. Frasch, FDA, Bethesda, Maryland). All antibody determinations were performed at 3-5 serial dilutions from which mean titres were calculated. The inter-assay variability for the above ELISA was 2-10%. An adequate antibody response was defined as a four-fold or greater increase in antibody levels in addition to a minimal titre of 50 U/ml corresponding to 18.8 microgram/ml and which is 50% of the titre in the reference serum.

Antibody avidity

Avidity indices of IgG anti-*Hib* antibodies were measured by a modification of the sodium thiocyanate (NaSCN) elution method described by Pullen *et al*[17,16]. Antibody avidity can only reliable be determined in sera with a minimal OD value of 1.0 at a 1:50 dilution, corresponding to a minimal *Hib* antibody concentration of 25 microgram/ml. Polystyrene microtitre plates were coated overnight at 37°C with *Hib* (HbO-HA Lot 17, Wyeth Lederle Vaccines, NISCB, UK) diluted to 1 microgram/ml in PBS. After blocking for 1 hour at 37°C with PBS-1% bovine serum albumin, serum samples and standard serum were incubated for 2 hours at 37°C. Sera were diluted in PBS-0.05% Tween 20-1% bovine serum albumin to an OD reading of 1.0. After washing, NaSCN diluted in PBS was added at concentrations ranging from 0.0 to 3.5 M. After 15 minutes at 37°C, the wells were washed, and binding of remaining antibodies was then detected by subsequent incubation with alkaline phosphatase-conjugated goat anti-human IgG (Biosource, Camarillo, CA). After incubation with substrate (*p*-nitrophenylphosphate, Sigma Chemical Co, St. Louis, MO), the reaction was stopped with 2.4 M NaOH, and the optical density at 405 nm was recorded. The relative avidity index (AI) is defined as the molarity of NaSCN at which 50% of the amount of IgG antibodies bound to the coated antigen in the absence of NaSCN has been eluded.

Phagocytosis

Phagocytosis-inducing capacity of anti-*Hib* IgG antibodies was determined by a modification of the method described by Sanders *et al*[18]. A *H. influenzae* type b strain (lot number 920029I, kindly provided by Dr. L. Spanjaard, National Reference Laboratory for Bacterial Meningitis, AMC, Amsterdam, the Netherlands) was cultured in brain-heart broth (Difco; Becton, Dickinson & Company, CA) supplemented with 1% haemin (Fluka, Buchs SG, Switzerland) and vitox (Oxoid, Hampshire, England) in a shaking waterbath at 37°C overnight. A series of dilutions was then grown on agar plates (chocolate Haemophilus agar plates, bioMérieux, Marcy l'Etoile, France) and the number of colony forming units (CFU) was determined. 1x 10^9 CFU per millilitre medium was then stored at -80 degrees Celsius until further use. For FITC labelling, 1x 10^9 bacteria were thawed and resuspended in 1 ml fluorescein isothiocyanate solution (FITC, Pierce Biotechnol-

ogy, Rockford, IL; 0.5 mg FITC per ml PBS). After incubation for 30 minutes at 37°C under constant gentle shaking, FITC-labelled bacteria were washed three times in PBS and stored in the dark at 4°C (for a maximal period of 4 weeks) until use.
All experiments were performed with polymorphic mononuclear cells (PMN) from donors with the FcγRIIa H/H phenotype. Heparinised peripheral blood was centrifuged over a ficoll-histopaque gradient, the cell pellet was washed and treated twice with erytrocyte lysing buffer on ice[10]. PMN's were counted and the sample was diluted in tissue culture medium (RPMI-10% foetal calf serum [FCS]) to a concentration of $5x\ 10^5$ PMN/ml. Preliminary experiments with addition of exogenous human complement (pooled fresh serum from 20 healthy adults) in a dose range of 2-10% (vol/vol) showed improved phagocytosis. However, under these conditions the phagocytosis was no longer antibody dependent (data not shown). Therefore, no exogenous complement source was added in the following experiments and serum samples were heat-inactivated. $8x\ 10^6$ CFU of FITC-labelled *Hib* were incubated with serum samples (inactivated for 30 minutes at 56°C and diluted 1:10 in RPMI-10% FCS) for 30 minutes in a shaking waterbath at 37°C. After washing, the opsonised *Hib* was incubated with $2x10^5$ PMN's (*Hib*: PMN ratio 40:1) for 7.5 minutes in a shaking waterbath at 37°C. In preliminary experiments in which incubation times were varied between 7.5 and 60 minutes, 7.5 minutes turned out to be optimal to detect differences between pre- and post-vaccination samples (data not shown). Phagocytosis was stopped by adding ice-cold buffer (PBS-1% BSA-0.01% FCS). After washing and resuspension, PMN's were kept at 4°C and flowcytometry (FACSCalibur, Becton Dickinson, San Jose, CA) was performed within 4 hours. In each sample, data from 10.000 cells were acquired. Granulocytes were gated according to their characteristic forward and side scatter pattern. Subsequently, logarithmic FITC intensity was plotted versus relative cell number. Phagocytosis-inducing capacity of antibodies was expressed as the mean FITC fluorescence intensity (MFI).

Statistical analyses
Differences between pre- and post-vaccination antibody levels, avidity and phagocytosis were assessed by using a Wilcoxon signed rank test. A *p*-value of <0.05 was considered statistically significant.

Results

Patients

Sixteen patients (11 male, 5 female) with a mean age of 57 years (range 43-68) were included. Thirteen patients suffered from multiple myeloma and three patients suffered from non-Hodgkin's disease. No major adverse reactions after vaccination were reported. Two patients had recurrence of tumour at 9 and 12 months after transplantation. Therefore, data concerning the third vaccination of these two patients were not included in the analysis.

Peripheral blood lymphocytes subsets at time of first vaccination, as a measure of the cellular immune status are given in *Table* 1. Twelve out of 16 patients had low levels of CD4-positive T cells, three of the patients had subnormal levels of B cells, whereas only one patient showed a low level of CD8-positive T cells. At time of first vaccination, all patients displayed a CD4:CD8 ratio of less than 1.0. At 14 months after aSCT, only one patient had a CD4:CD8 ratio greater than 1.0 (data not shown).

Table 1. Lymphocytes subsets

	CD4 x 10^9/l	CD8 x 10^9/l	CD19 x 10^9/l
mean levels (range)	0.24 (0.12-0.5)	0.56 (0.14-1.1)	0.17 (0-0.4)
normal values¶	0.7 (0.3-1.4)	0.4 (0.2-1.2)	0.2 (0.1-0.5)

Mean levels (range) of lymphocytes subsets at time of first vaccination (i.e. 6 months after transplantation) are shown. ¶ Normal values (median, 5th and 95th percentile) are from Comans-Bitter *et al*[19].

Antibody responses

IgG antibody titres against *Hib* are depicted in *Figure* 1. Mean anti-*Hib* IgG antibody titres significantly increased from 1.1 microgram/ml before vaccination to 87 and 489 microgram/ml after two and three vaccinations, respectively (*p*-value 0.001 and 0.001, respectively). After three vaccinations, a sufficient antibody response to *Hib* vaccine (arbitrarily defined as a four-fold or higher increase in specific IgG anti-*Hib* titres in addition to post-vaccination levels of at least 50 U/ml corresponding to 18.8 microgram/ml) was found in all but one of the 14 eligible patients. This patient did not acquire anti-*Hib* antibodies after any of the three vaccinations.

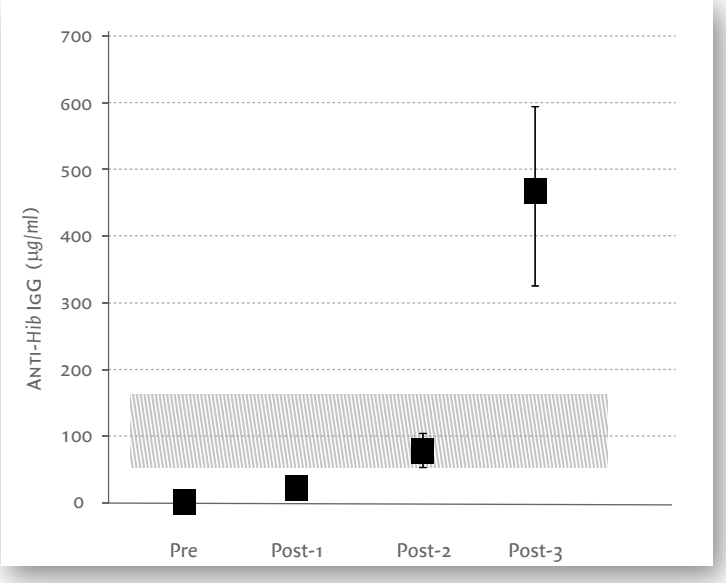

Figure 1. Anti-*Hib* IgG antibodies of 14 patients before (pre) and after one (post-1), two (post-2) and three (post-3) vaccinations with conjugated *Hib* vaccine. Anti-*Hib* antibodies increased significantly after three vaccinations (*p*-value 0.001). Hatched area indicates mean value (± SE) of 12 healthy adults vaccinated with a single dose of *Hib* conjugate vaccine.

Antibody avidity and phagocytosis

In order to study the functional capacity of antibodies we performed NaSCN elution to determine avidity. Preliminary experiments showed that addition of NaSCN does not affect the amount of polysaccharide bound to the plate (data not shown). Avidity indices (i.e. the molarity of NaSCN at which 50% of the amount of IgG antibodies bound to the coated antigen in the absence of NaSCN has been eluded from the antigen) are depicted in *Figure 2*. Mean AI increased significantly from 0.57 after two vaccinations to 1.22 after three vaccinations (*p*-value 0.047). After repeated vaccinations, avidity of anti-*Hib* IgG antibodies increased in all but one patients who were eligible for avidity determination. This patient did show an increase in anti-*Hib* antibodies after all vaccinations, but after the third vaccination the avidity index decreased.

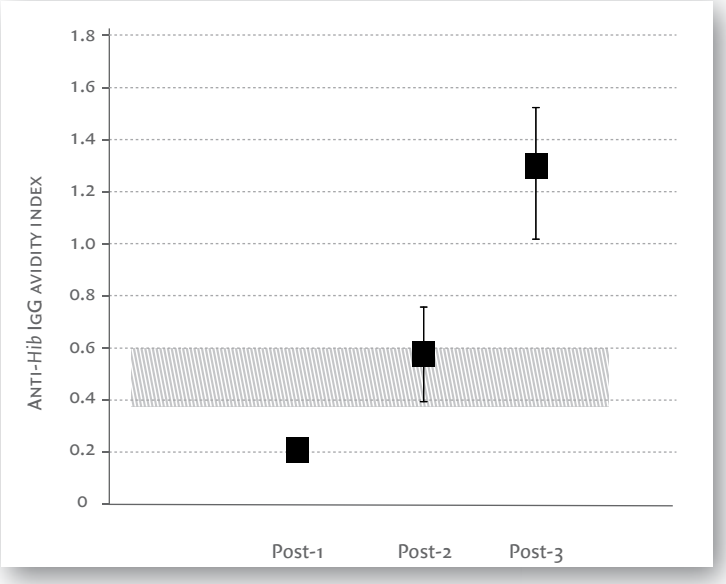

Figure 2. Anti-*Hib* IgG antibodies avidity indices (AI) after one (post-1), two (post-2) and three (post-3) vaccinations with *Hib* vaccine. Mean AI plus standard errors are shown. Mean AI increased significantly after three vaccinations with conjugated *Hib* vaccine, as compared with AI after two vaccinations (*p*-value 0.047). Hatched area indicates mean value (± SE) of 12 healthy adults vaccinated with a single dose of *Hib* conjugate vaccine.

Subsequently, opsonizing activity of antibodies was determined by a phagocytosis assay. Phagocytosis-inducing capacity of anti-*Hib* IgG antibodies (expressed as the mean FITC fluorescence intensity, MFI) is shown in *Figure 3*. Mean MFI increased significantly after two and three vaccinations (*p*-value 0.03 en 0.002, respectively). As could be expected, the previously mentioned patient who did not acquire anti-*Hib* antibodies did not show an increase in phagocytosis-inducing capacity. One other patient, who did have a high increase in antibodies but a decrease in avidity index, also showed a decrease in MFI after the third vaccination.

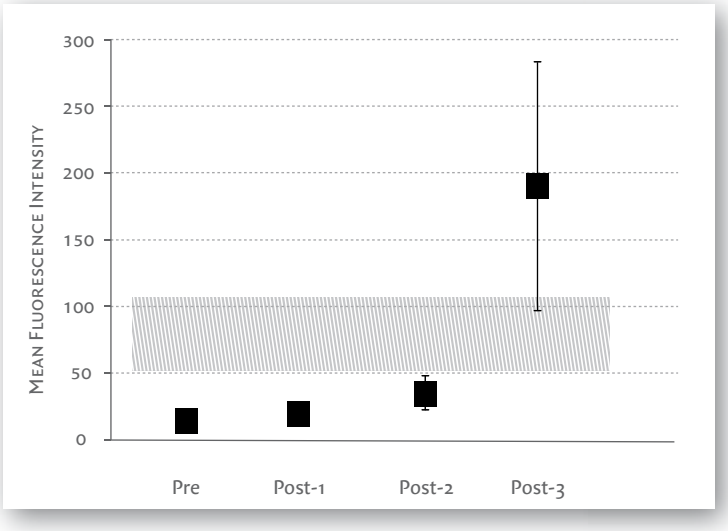

Figure 3. Phagocytosis-inducing capacity of anti-*Hib* IgG antibodies of 14 patients, expressed as mean fluorescence intensity (MFI), before and after one, two and three vaccinations with *Hib* vaccine. MFI plus standard errors are shown. MFI increased significantly after two and three vaccinations, as compared with MFI before vaccination (*p*-values 0.03 and 0.002, respectively). Hatched area indicates mean value (± SE) of 12 healthy adults vaccinated with a single dose of *Hib* conjugate vaccine.

Discussion

In this study, antibody titres, avidity and phagocytosis-inducing capacity after multiple vaccinations with conjugated *Hib* vaccine are described in patients who underwent aSCT. Significant increases in anti-*Hib* IgG antibody titres were found after two and three vaccinations. A response rate of 38%, 75% and 93% was achieved after one, two and three vaccinations, respectively. These results are consistent with other studies. In patients suffering from multiple myeloma who received a single *Hib* vaccination, Robertson *et al*[20] found a four-fold increase in antibody titres in 41% of 44 patients. In allogeneic and autologous bone marrow transplant recipients, 80-89% of all patients achieved protective antibody levels after two, three or four doses *Hib* vaccine[9].
Although antibody titres are often considered adequate predictors of vaccine efficiency, anti-*Hib* antibody levels decline gradually after vaccination[21]. Functional parameters of antibodies such as antibody avidity and phagocytosis-inducing ca-

pacity provide additional information regarding the effects of vaccination. For instance, an increase in antibody avidity during the year following *Hib* vaccination with a concurrent decrease in antibody levels has been described in children[21]. Higher antibody avidity has been shown to improve bactericidal activity in vitro[22]. In bone marrow transplant recipients, only phagocytic activity against pneumococcal polysaccharides has been studied so far[14].

We measured anti-*Hib* antibody avidity and phagocytosis-inducing capacity. In most patients, after three vaccinations, an increase in anti-*Hib* antibody levels was accompanied by an increase in avidity and phagocytosis-inducing capacity of antibodies. Two patients had striking outlying results. One patient did not acquire anti-*Hib* antibodies even after three vaccinations. Because of absent antibodies, antibody avidity could not be determined in this patient. As could be expected, phagocytosis-inducing capacity remained low during the whole study period. The absence of antibody formation in this patient might be partly due to a very low relative CD19-count in the circulation (<1%) at the time of the first vaccination, also reflected in low serum IgG, IgM, IgG1 and IgG2 levels. In the other patient, a third vaccination with *Hib* resulted in an increase in anti-*Hib* antibody titre from 68 microgram/ml to 305 microgram/ml. However, repeated experiments showed a decrease in antibody avidity and phagocytosis-inducing capacity after this vaccination. We have no ready explanation for this exceptional response pattern. Potentially, non-IgG antibodies, such as IgA could block phagocytosis. We therefore also determined the IgA anti-*Hib* antibody response. Indeed, this patient showed a robust IgA response after the third *Hib* vaccination (a rise from 163 to 2034 U/ml), which was higher than in the other patients (range 1 to 643 U/ml after 3 *Hib* vaccinations). However, the high IgA antibody response cannot readily explain the drop in IgG avidity, apart from the theoretical possibility that all high affinity IgG-bearing B lymphocytes would have switched to IgA. Therefore, although the reason for the decline in avidity is unknown, the data do underline the relation between antibody avidity and opsonophagocytosis. Furthermore, it shows that an increase in antibody quantity is not always accompanied by an increase in antibody quality.

With regard to the avidity assay, the sensitivity of the assay limited the measurements of avidity in sera with low antibody levels. Moreover, we determined antibody avidity three weeks after vaccination. Goldblatt *et al* described further avidity maturation one year after primary immunization with *Hib* vaccine in children[21]. Therefore, it is possible that further increases in avidity may take place after the

follow up period in the current study.
Our data show that a vaccination scheme starting at 6 months after aSCT can induce an antibody response with avidity maturation and functional support of *Hib* phagocytosis. During the series of 3 vaccinations, spanning the period between 6 to 14 months post aSCT, there is ongoing reconstitution of the patients' immune system in general. The functional capacity of the antibodies, therefore, is the net effect of both vaccination and ongoing reconstitution.
In this series of patients, we observe differences in the kinetics of maturation of the anti-*Hib* antibody response. A single patient even totally lacked an anti-*Hib* response. Design of the optimal post-aSCT vaccination scheme will require a better insight of the factors that govern the reconstitution of humoral immune responsiveness. We therefore have started a study in which we will analyze the in vitro T-lymphocyte response and cytokine profile to this vaccination scheme.
In conclusion, in this study with autologous stem cell transplant recipients, multiple vaccinations with a conjugated *Hib* vaccine resulted in high antibody levels and maturation of antibody functionality.

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CHAPTER 5

Vaccination responses and lymphocyte subsets after autologous stem cell transplantation

Vaccination responses and lymphocyte subsets after autologous stem cell transplantation

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Submitted

Summary

Twenty autologous stem cell transplant recipients were vaccinated with 3 doses of Diphtheria-Tetanus-Poliomyelitis vaccine and conjugated *Haemophilus influenzae* type b (*Hib*) vaccine. Pneumococcal vaccination consisted of 2 doses of conjugated vaccine followed by a single dose of non-conjugated polysaccharide vaccine, at six, eight and fourteen months after transplantation, respectively. Mean anti-tetanus, anti-*Hib* and anti-pneumococcal IgG antibodies significantly increased after each vaccination. Response rates after the full vaccination schedule were 94% for *Hib* and 72% for both conjugated 7-valent and non-conjugated 23-valent pneumococcal vaccine. Three months after transplantation, $CD16^+CD56^+$NK cells were in the normal range and remained so. The total number of T lymphocytes at 3 months was and remained in the normal range. The mean CD4/CD8 ratio was 0.43 at 3 months post aSCT and, while gradually increasing, remained subnormal. The mean number of $CD19^+$ B lymphocytes significantly increased during the study period. Patients with CD19 counts $\leq 0.10 \times 10^9$/L required at least two *Hib* vaccinations to show a response, while patients with CD19 counts $\geq 0.20 \times 10^9$/L showed a response to *Hib* after one vaccination only. Thus, a minimum threshold level of $CD19^+$ cells appears to be required for adequate responses to vaccination.

Introduction

Autologous stem cell transplantation (aSCT) has become a common procedure in the treatment of patients with haematological malignancies such as multiple myeloma and non-Hodgkin's disease[1]. The conditioning regimen for aSCT leads to significant impairment of the immune system of the host and although infectious complications are less frequent as in allogeneic transplantation, it is also a major source of morbidity in patients undergoing autologous stem cell transplantation[2,3]. Post-transplantation bacterial pneumonia is often caused by encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (*Hib*)[2,3]. Vaccination of stem cell transplant recipients with, amongst others, pneumococcal vaccine and *Hib* vaccine has been recommended by institutions such as the Centers for Disease Control and Prevention, the American Society of Hematology and the European Group for Blood and Marrow Transplantation[4,5,6]. Several previous studies have described the antibody responses to vaccination with *Hib*, tetanus and pneumococcal polysaccharide[7,8,9,10]. In most of these studies, adequate antibody responses were found after repeated vaccinations with *Hib*-conjugate vaccine, but the antibody responses to pneumococcal polysaccharide vaccines, even in conjugated form, were generally less.
After aSCT, recovery of the innate immunity is usually seen within weeks[11]. On the contrary, adaptive immunity takes much longer to recover. The ability to generate a quantitative and qualitative sufficient antibody response depends on a functional B-lymphocyte and T-lymphocyte compartment. Reconstitution of lymphocyte subsets after aSCT has been studied by several groups[12,13,14]. T-cell dependent antibody responses can be subnormal for months to years, whereas T-cell independent antibody responses take much longer to recover.
We conducted a prospective follow-up study to determine the antibody responses to multiple vaccinations with DTP vaccine (Diphtheria, Tetanus and Poliomyelitis), conjugated *Haemophilus influenzae* type b vaccine and pneumococcal vaccines in autologous stem cell recipients. The antibody response was evaluated in the context of the cellular immune reconstitution, assessed by determination of the major lymphocyte subsets in blood.

Materials and methods

Patients and vaccination schedule

Adult patients with non-Hodgkin's lymphoma, multiple myeloma or amyloidosis who underwent aSCT, were included. Exclusion criteria were vaccination with DTP, *Hib* or pneumococcal vaccine in the first three months after transplantation and recurrence of tumour or start of chemotherapy within six months after aSCT. All patients gave written informed consent. The study was approved by the Medical Ethical Committees of participating hospitals.

Six months after aSCT, patients were vaccinated with DTP vaccine (Diphtheria, Tetanus and Poliomyelitis vaccine, National Institute of Public Health and the environment, RIVM, Bilthoven, The Netherlands), *Hib* (PRP-T vaccine, polyribosylribitolphosphate (PRP) conjugated to tetanus toxoid (TT), Pasteur Mérieux Sérums et Vaccins, Lyon, France) and the diphtheria CRM_{197} protein conjugated heptavalent polysaccharide pneumococcal vaccine Prevnar (Prevnar®, Wyeth Lederle Vaccines and Pediatrics, Rochester, NY). These vaccinations were repeated two months later, i.e. 8 months after aSCT. At 14 months after transplantation, patients received a booster with DTP, *Hib* and the non-conjugated polysaccharide pneumococcal vaccine Pneumo 23 (Pneumo 23®, Aventis Pasteur MSD, Rahway, NJ). All vaccines were administered in the upper arms intramuscularly. Patients were being observed fifteen minutes after each vaccination and were asked to record local and systemic reactions on a case report form during three days after vaccination.

Antibody levels

IgG antibody levels to tetanus toxoid (TT), *Haemophilus influenzae* type b and to pneumococcal polysaccharides of serotypes 6B, 9V and 19F (included in Prevnar®) as well as 1, 11A and 15B (included in Pneumo 23®) were measured by ELISA as described previously[15, 16]. Serum samples were taken before the first vaccination and 3 weeks after each vaccination. Pre- and post-vaccination serum samples from individual patients were analysed on the same ELISA plate. All antibody determinations were performed using 3-5 serial dilutions from which mean titres were calculated. The inter-assay variability for the above ELISAs was 2-10 %. For the pneumococcal polysaccharide antibody assay, serum samples were pre-incubated with excess (50 microgram/ml) pneumococcal common cell wall polysaccharide (CPS) overnight at 4°C before analysis to block anti-CPS antibodies[17]. Anti-TT IgG antibodies were expressed in IU/ml, relative to standard serum.

Anti-*Hib* and anti-pneumococcal IgG antibodies were expressed in micrograms/ml, relative to standard serum (human *Haemophilus* reference serum, lot 1983, and human pneumococcal reference serum, lot 89-SF, kindly provided by Dr. C. Frasch, FDA, Bethesda, Maryland). All antibody titres were log-transformed and geometric mean titres (GMT) calculated. For statistical analysis, antibody titres that were less than the limit of the assay's sensitivity were assigned values equal to one-half of the lower detection limit. Data are also reported in terms of proportions of patients who showed response (defined as a ≥ 4-fold increase in antibody titre) and who attained protective antibody titres. For *Hib*, anti-PRP antibody concentrations ≥ 1.0 µg/mL are considered to correlate with long-term protection[18], whereas for tetanus, antibody concentrations of 0.1 IU/mL are correlated with long-term protection[19]. For *Streptococcus pneumoniae*, anti-pneumococcal antibody titres of ≥ 0.35 µg/mL have been recommended by the WHO as an estimate of the threshold concentration for protection against invasive disease[20].

Immunophenotyping

Heparinised peripheral blood samples were obtained at three, six, eight and fourteen months after stem cell transplantation. PMNL were isolated by a ficoll-isopaque gradient centrifugation and stored in 10% DMSO at -80 ºC until use. Samples from individual patients were thawed and stained for flowcytometry in a single run. The following monoclonal antibodies were used: anti-CD16-56 (phycoerythrin, PE), anti-CD19 (allophycocyanin, APC), anti-CD3 (Peridinin chlorophyll protein, PerCP), anti-CD4 (APC), anti-CD8 (PE), anti-CD45RA (fluorescein isothiocynate, FITC) and anti-CD45RO (PE). The monoclonal antibodies were added to 100 microliter cell suspension and incubated for 30 minutes at room temperature. After washing, cells were resuspended in 200 microliter phosphate-buffered saline and flowcytometric analysis (FACSCalibur, Becton Dickinson, San Jose, CA) was performed within 4 hours.

Statistical analyses

Comparisons of GMT before and after vaccination (paired samples) were performed using the Wilcoxon signed rank test; comparisons between 2 groups (unpaired samples) were performed using the Mann-Whitney U test. For categorical variables, univariate, between groups, comparison was performed by means of the Chi-square test.

Results

Patients

Twenty patients (15 male, 5 female) with a mean age of 57 years (range 43-68) were included. The haematological diagnosis was multiple myeloma in fifteen patients, amyloidosis in two patients and non-Hodgkin's disease in three patients. Two patients (both suffering from multiple myeloma) developed recurrence of disease, necessitating chemotherapy, and were subsequently excluded from analysis. Patients with multiple myeloma or amyloidosis received high dose melphalan (cumulative dose 200 mg/m^2), whereas patients with NHL received the BEAM-regimen (cumulative doses: BCNU 300 mg/m^2, ARA-C 800 mg/m^2, VP16 800 mg/m^2, melphalan 140 mg/m^2) as conditioning therapy. The patients were transplanted with a mean of 7.93 x10^6 CD34$^+$ cells per kg body weight. After vaccination, no major adverse reactions were reported.

Antibody responses

IgG antibody titres against TT, *Hib* and pneumococcal polysaccharides are depicted in *Figure* 1. Geometric mean anti-TT IgG antibodies significantly increased from 0.1 IU/ml to 0.5, 2.7 and 10.2 IU/ml after each vaccination (*p*-values < 0.005). Geometric mean anti-*Hib* IgG antibody titres also significantly increased from 0.3 mg/ml before vaccination to 12.6 and 57.7 mg/ml after two and three vaccinations, respectively (*p*-values < 0.005). For each pneumococcal serotype, the geometric mean IgG antibody titre increased significantly after each vaccination (*p*-values <0.02), except for anti-PPS19F, which showed significant increases only after two and three vaccinations.

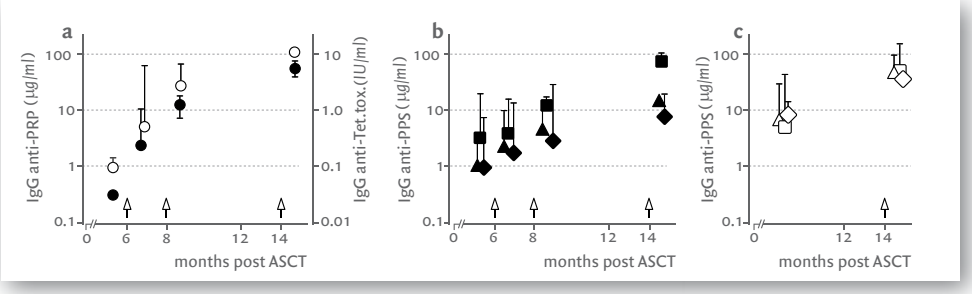

Figure 1. Antibody response after vaccination. A: IgG antibodies against the capsular polysaccharide of *Haemophilus influenzae* type b (Hib) polyribosyl ribitolphosphate (PRP; closed circles, expressed in mg/ml) and Tetanus toxoid (TetTox; open circles, expressed in IU/ml) were determined before and after each vaccination (indicated with open arrows). B: IgG antibodies against pneumococcal polysaccharide (PPS) serotype contained within the conjugate vaccine, 6B (closed triangles), 9V (closed diamonds), and 19F (closed squares). C: IgG antibodies against PPS serotypes contained within the pneumococcal polysaccharide vaccine, 1 (open squares), 11A (open triangles), and 15B (open diamonds). Data shown are geometric mean titers and the upper limit of the 95% confidence interval. Anti-PRP (n=18) and anti-TetTox (n=14) antibodies increased significantly after each vaccination (p-values <0.005). The geometric mean IgG antibody titres for each pneumococcal serotype increased significantly after each vaccination (p-values <0.02), except for anti-PPS19F, which showed significant increases only after two and three vaccinations.

For *Hib*, response rates were 56, 83 and 94% after one, two and three vaccinations, respectively. The percentage of patients with anti-*Hib* antibody levels above the protective threshold increased from 17% before the first vaccination to 94% after the full vaccination schedule. For TT, response rates were 43, 86 and 93% after one, two and three vaccinations, respectively. The percentage of patients with anti-TT antibody levels above the protective threshold of 0.1 iU/ml increased from 50% before vaccination to 100% after the full vaccination schedule. After one and two doses of Prevnar®, response rates were 6 and 39%, respectively. After the booster vaccination with Pneumo 23®, the response rate to the conjugate serotypes increased to 72%, whereas 72% of the patients showed a response to the non-conjugate pneumococcal serotypes. After the full vaccination schedule, the percentage of patients with antibody levels above the protective threshold was 83% for the conjugated pneumococcal vaccine and 72% for the non-conjugated pneumococcal vaccine.

Cellular immunoreconstitution

Results of immunophenotyping of peripheral blood lymphocytes are given in *Table 1*. The numbers of T lymphocytes as well as of $CD16^+CD56^+$ NK cells were already within the normal range at three months after aSCT. The numbers of $CD3^+$ cells remained at the lower end of the normal range during the whole study period. Mean counts of $CD19^+$ cells were at the lower normal range (0.16 $x10^9$/L) at three months after aSCT and 4 out of 18 patients had decreased numbers of $CD19^+$ cells. In the period thereafter $CD19^+$ cells significantly increased to 0.22 and 0.34 $x10^9$/L at six and fourteen months after aSCT, respectively (*p*-values < 0.025). In one patient $CD19^+$ cells remained undetectable during the whole study period. This patient showed a negative antibody response to *Hib* and pneumococcal vaccination. All other patients with $CD19^+$ counts $\leq 0.10x10^9$/L before vaccination needed at least two vaccinations to show a response to *Hib*, all patients with $CD19^+$ counts $\geq 0.20x10^9$/L showed a response after only one vaccination. The CD4/CD8 ratio was persistently below normal values, but increased from 0.43 at three months after aSCT to 0.54 and 0.71 at six and fourteen months after aSCT, respectively (*p*-values < 0.03). There was no relation between the CD4/CD8 ratio and the antibody response to any of the vaccines. Numbers of $CD45RO^+$ and $CD45RA^+$ T cells did not change significantly during the study period. At six months after stem cell transplantation (i.e. before the first vaccination), the percentage of $CD45RO^+$ cells was already in the normal range. The percentage $CD45RA^+$ cells was persistently below normal values. Also here no relation was found between CD45R isoform expression on T lymphocytes and vaccination induced antibody response. No significant differences in lymphocyte subsets were found between patients with non-Hodgkin's lymphoma, multiple myeloma or amyloidosis (data not shown).

Table 1. Lymphocyte subsets. Absolute counts of $CD3^+$, $CD19^+$ and $CD16^+CD56^+$ cells, CD4/CD8 ratio and percentages of $CD45RA^+$ and $CD45RO^+$ cells at different time points after aSCT are shown (mean levels ± SE). Normal values (median, minimal and maximal values) are from de Vries *et al*[21].

Lymphocyte subsets	3 months	6 months	14 months	normal values
$CD3^+$ ($x10^9$/L)	0.79 ± 0.07	0.83 ± 0.08	0.98 ± 0.14	1.5 (0.7-1.8)
$CD19^+$ ($x10^9$/L)	0.16 ± 0.05	0.22 ± 0.06	0.34 ± 0.06	0.2 (0.1-0.4)
$CD16^+CD56^+$ ($x10^9$/L)	0.21 ± 0.04	0.15 ± 0.02	0.22 ± 0.03	0.2 (0.1-0.4)
CD4/CD8 ratio	0.43 ± 0.07	0.54 ± 0.06	0.71 ± 0.12	2.0 (1.2-2.7)
$CD45RA^+$ (% of $CD3^+$)		30 ± 6.8	27 ± 4.2	60 (46-71)
$CD45RO^+$ (% of $CD3^+$)		62 ± 6.8	65 ± 4.9	56 (48-71)

Discussion

In this study, the antibody response after multiple vaccinations with DTP, conjugated *Hib* vaccine and pneumococcal vaccines are described in patients who underwent aSCT. Significant increases in IgG antibody titres were found after vaccination. For *Hib*, a response rate of 56, 83 and 94% was achieved after one, two and three vaccinations, respectively. These results are consistent with other studies: in patients suffering from multiple myeloma who received a single *Hib* vaccination, Robertson *et al* found a fourfold increase in antibody titres in 41% of the patients[8]. In allogeneic and autologous bone marrow transplant recipients, 80-89% of all patients achieved protective antibody levels after two, three or four doses *Hib* vaccine[7]. In our study, antibodies to TT increased significantly after each vaccination. However, 50% of the patients had anti-TT IgG antibodies above the protective level (i.e. > 0.1 IU/ml) already before the first vaccination.
Non-conjugated polysaccharide vaccines elicit a T-cell independent immune response and have been shown to be ineffective in immature immune systems such as seen in children or after stem cell transplantation[22]. A heptavalent conjugated pneumococcal polysaccharide vaccine has been developed which induces a T-cell dependent antibody response. In children, vaccination with pneumococcal conjugate vaccine followed by pneumococcal non-conjugated vaccine results in strong antibody responses[23]. Therefore, we vaccinated patients with a conjugated pneumococcal vaccine twice, followed by a single booster vaccination with a 23-valent non-conjugated polysaccharide vaccine after 6 months. Booster vaccination with the polysaccharide vaccine not only serves to induce higher antibody levels but also offers broader serotype coverage than conjugate vaccination only. In our study, the conjugated pneumococcal vaccine resulted in a modest increase in antibody levels as compared to the conjugated *Hib* vaccine. These results are consistent with a study in patients with Hodgkin's disease, in which also a lower antibody response to a heptavalent pneumococcal conjugate vaccine compared to anti-*Hib* conjugate was found[24]. However, a single vaccination with the non-conjugated vaccine resulted in a response rate of 72%, which is comparable to response rates in elderly[25]. These data fit into the hypothesis, that one year post-transplantation, non-conjugated vaccines can be effective since T-cell independent immune responses might be restored. It should be kept in mind however that the priming doses with conjugate vaccine may have primed the polysaccharide specific B lymphocytes.

We analysed the cellular immune reconstitution after aSCT by determination of lymphocyte subsets at different time points after transplantation. As described by others[11], recovery of the innate immune system (in our study measured by $CD16^+CD65^+$ cells) was seen early after aSCT. During the whole study period, an inversed CD4/CD8 ratio was seen, which is in accordance with previous studies[12,13,26]. Despite the persistent inversed CD4/CD8 ratio, adequate responses to *Hib*, TT and pneumococcal vaccination were found in our patients. During the study period, mean levels of $CD19^+$ cells significantly increased. $CD19^+$ cells obviously are required for antibody production in response to vaccination, but whether peripheral blood is a representative compartment for measurement of B-lymphocyte status is uncertain. Yet, the single patient who did not develop peripheral blood $CD19^+$ cells at any time during the study period did not respond to *Hib* or pneumococcal vaccination. Moreover, all patients with $CD19^+$ cell counts of $\leq 0.10 \times 10^9$/L, needed at least two *Hib* vaccinations to show a response, while all patients with $CD19^+$ cell counts $\geq 0.20 \times 10^9$/L showed a response to *Hib* after one vaccination only. Thus, the number of $CD19^+$ cells in peripheral circulation may serve as a correlate for an adequate antibody response after vaccination.
In accordance with earlier studies[12,13,26], we also found persistently low levels of $CD45RA^+$ T cells during the whole study period, while $CD45RO^+$ counts were already in the normal range at six months after transplantation. $CD45RO^+$ cells correspond to mature T cells that are able to respond to antigenic stimulation, while $CD45RA^+$ cells are naïve and/or immature cells. $CD45RO^+$ cells reconstitute via thymus-independent pathways, while $CD45RA^+$ cells follow T-cell ontogeny[12,13,26]. Because of age and chemotherapy, residual thymus function is expected to be low in our patients, thus making the slow recovery of $CD45RA^+$ cells not surprisingly.
It is widely recognized that the period of immunodeficiency, early after aSCT poses a risk for infectious complications of this procedure. Immunisation against vaccine-preventable diseases is hampered by poor B- and T-lymphocyte function. Recently, a strategy has been developed that involves pre-transplant vaccination followed by ex vivo co stimulation of autologous T lymphocytes which are reinfused post-transplantation[27]. This procedure allows for (re)vaccination with pneumococcal conjugate vaccine at 1 and 3 months post aSCT and results in better antibody responses than control groups without T-lymphocyte infusion or without pre-transplant vaccination. Even with such a demanding procedure, up to 30% of patients shows insufficient responses to pneumococcal serotypes

6B and 14[27]. Our vaccination schedule, starting at 6 months after aSCT provides nearly complete protection for *Hib* and TT (and presumably other T-cell dependent protein antigens as well) and a sufficient response to the prevalent pneumococcal serotypes in most patients. Since a minimum threshold level of $CD19^+$ cells appears to be required for adequate responses to vaccination, we recommend monitoring of the number of B lymphocytes before vaccination.

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CHAPTER 6

Development of T-cell mediated immunity after autologous stem cell transplantation: prolonged impairment of antigen-stimulated production of γ-interferon

Development of T-cell mediated immunity after autologous stem cell transplantation: prolonged impairment of antigen-stimulated production of γ-interferon

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Summary

The conditioning regimens for autologous stem cell transplantation (aSCT) lead to impairment of the immune system and concomitant increase in susceptibility to infections. We studied recovery of cellular immunity by in vitro analysis of T-cell proliferation and cytokine production profiles during the first 15 months after aSCT in patients with multiple myeloma and non-Hodgkin's lymphoma. Peripheral blood mononuclear cells (PBMC) were collected at 6, 9 and 15 months after transplantation and stimulated with a combination of CD2 and CD28 monoclonal antibodies, with PHA or with tetanus toxoid as recall antigen. A multiplex enzyme linked immunoassay was used to determine levels of Th1 cytokines IL-2, IFN-γ and TNF-α, Th2 cytokines IL-4, IL-5 and IL-13, the regulatory cytokine IL-10, the pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and the chemokine IL-8. T-cell proliferation progressively increased from 6 to 15 months after aSCT. Overall, cytokine production increased after aSCT. Production of Th2 cytokines IL-5 and IL-13 was superior to production of Th1 cytokines IFN-γ and TNF-α. We hypothesise that prolonged impairment of IFN-γ production might contribute to the relatively high incidence of viral infections after aSCT.

Introduction

High dose chemotherapy followed by autologous peripheral stem cell transplantation (aSCT) is a widely used therapy for many haematological malignancies[1,2]. High dose chemotherapy destroys malignant cells, whereas stem cell transplantation rescues the otherwise fatal pancytopenia. Although peripheral stem cell transplantation is believed to shorten the early period of severe neutropenia as compared to bone marrow transplantation[3], the functional capacity of the immune system remains partially impaired for a year or even longer after transplantation[4,5,6]. The impairment of the immune system in the first period after autologous stem cell transplantation is reflected in the high incidence of infectious complications, which is a major source of morbidity[7,8]. B-lymphocyte numbers in peripheral blood normalise within 6-12 months after aSCT, but the capacity to mount an adequate antibody response to vaccination is low until 6 months after transplantation and gradually improves thereafter[9,10]. Several studies on the quantitative development of cellular immunity after aSCT found normal or even high levels of natural killer (NK) cells 2 weeks after transplantation already[4,5,6]. T-lymphocyte numbers, however, remain low for a substantial longer period[11]. High dose chemotherapy will destroy most T cells in the recipient. Following transplantation, stem cells present in the graft, will develop into mature T cells as in normal ontogeny including maturation in the thymus[5,6]. Thymus function in these patients is reduced because of age and previous chemotherapy and therefore, the functional maturation of T cells will take a long time. We studied development of T-cell function in autologous stem cell transplant recipients during the first 15 months after transplantation. In vitro T-cell proliferation and the cytokine production profile were determined in this prospective cohort study.

Materials and methods

Patients

Adult patients with non-Hodgkin's lymphoma or multiple myeloma and who underwent aSCT, were included. The study was performed in the context of a larger study on the immune response to vaccination in autologous stem cell recipients as reported previously[9]. In short, patients were vaccinated at 6, 8 and 14 months after aSCT with Diphtheria, Tetanus and Poliomyelitis (DTP) vaccine (3 doses), tetanus toxoid (TT) conjugated *Haemophilus influenzae* (*Hib*) vaccine (3 doses) and

a combination of pneumococcal conjugate vaccine (2 doses) and pneumococcal polysaccharide vaccine (1 dose). Patients with recurrence of tumour or re-introduction of chemotherapy within six months after aSCT were excluded. All patients gave written informed consent. The study was approved by the Medical Ethical Committees of the participating hospitals.
The normal controls consisted of healthy hospital personnel aged 28-61 years (median 37 years). All of the controls were vaccinated during childhood with tetanus toxoid as part of the national childhood vaccination schedule.

T-cell proliferation, cytokine determination and lymphocyte subsets
Blood samples were collected at 6, 9 and 15 months after transplantation, which was before, after two and after three vaccinations, respectively. Heparinised peripheral blood was diluted 1:1 in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) containing L-glutamine (2 mM) (Invitrogen Life Technologies), penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen Life Technologies) and supplemented with 10 % heat-inactivated fetal calf serum (FCS) and mononuclear cells were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The cells were washed two times, mixed with 10% DMSO (1:2 vol/vol) and stored frozen at -80°C until use. Mononuclear cells (0.4 x 10^5) were cultured in triplicate in 200 µl volumes (RPMI 1640 supplemented with 20% (vol/vol) heat-inactivated human AB serum) in 96 well round-bottom culture plates (Nunc, Wiesbaden, Germany) at 37 °C in 5% CO_2 with 100% relative humidity. Cells were stimulated with phytohaemagglutinin (PHA; 35 µg/ml), with a combination of 0.4 µg/ml anti-CD2 and 1 µg/ml anti-CD28 monoclonal antibodies (mAbs) (both obtained from BD Pharmingen, Erembodegem, Belgium), or with 1.5 µg/ml tetanus toxoid (National Institute for Public Health and the Environment, RIVM, Bilthoven, The Netherlands). Cells were cultured for 6 days, the last 12 hours in the presence of 0.5 µCi (19 kBq) ^3H thymidine (ICN Biomedicals, Amsterdam, The Netherlands). Cells were harvested by standard procedures and incorporated radioactivity was measured by liquid scintillation counting and expressed as counts per minute (cpm). Directly before addition of ^3H thymidine, 50 µl culture supernatant was removed and stored at -80 °C.
Cytokines were detected in supernatants by applying a multiplex immunoassay (Bioplex 100, Biorad, Hercules, CA, USA) using a procedure that has been described in detail previously[12]. For our purpose, IL-1α, IL-1β, IL-6 and IL-8 (CXCL-

8) were measured as pro-inflammatory cytokines and chemokines, IL-2, IFN-γ and TNF-α as Th1 cytokines. Furthermore, IL-4, IL-5 and IL-13 were measured as representative Th2 cytokines and IL-10 as a regulatory cytokine[13,14].
Lymphocyte subsets were determined by immunophenotyping using the following mAbs: anti-CD3 (conjugated to Peridinin chlorophyll protein, PerCP), anti-CD4 (Allo phycocyanin, APC), anti-CD8 (phycoerythrin, PE), anti-CD45RA (fluorescein isothiocynate, FITC) and anti-CD45RO (PE). The mAbs were added to 100 µl cell suspension and incubated for 30 minutes at room temperature. After washing, cells were resuspended in 200 µl phosphate-buffered saline and flowcytometric analysis (FACSCalibur, Becton Dickinson, San Jose, CA, USA) was performed within 4 hours.

Statistical analyses
Differences between pre- and post-vaccination cytokine levels and thymidine incorporation were assessed by using a Wilcoxon signed rank test. A *p*-value of <0.05 was considered statistically significant.

Results

Patients
Fifteen patients (10 male, 5 female) with a mean age of 58 years (range 43-68) were included. Haematological diagnosis was multiple myeloma in twelve patients and non-Hodgkin's lymphoma in three patients. Two patients (both suffering from multiple myeloma) developed recurrence of disease, necessitating chemotherapy, and were subsequently excluded from analysis. The study was performed in three teaching hospitals in The Netherlands. Identical mobilisation and conditioning protocols were used at each study location. Stem cell mobilisation was performed with CAD (cyclophosphamide, adriamycin and dexamethasone) plus G-CSF for patients with multiple myeloma and with DHAP (cisplatin, cytarabine and dexamethasone) plus G-CSF for patients with NHL. Patients with multiple myeloma received high dose melphalan (cumulative dose 200 mg/m^2) as conditioning therapy, whereas patients with NHL received the BEAM-regimen (cumulative doses: BCNU 300 mg/m^2, ARA-C 800 mg/m^2, VP16 800 mg/m^2, melphalan 140 mg/m^2). The patients were transplanted with a mean of 9.61×10^6 CD34$^+$ cells per kg body weight (range: 2.40-34.90). The autologous stem cell grafts were unselected. After stem cell transplantation, no G-CSF was administered.

Reconstitution of cellular immunity was studied by quantitative and qualitative T-lymphocyte analysis. At 6 months after aSCT, levels of $CD3^+$ T lymphocytes were within the normal range. The mean CD4/CD8 ratio was 0.45 at 6 months and, although consistently increasing, remained subnormal during the whole study period. Moreover, while levels of $CD45RO^+$ cells were in the normal range at 6 months post-transplantation, levels of $CD45RA^+$ cells were persistently low at 6 and 15 months after transplantation *(Table 1)*.

Table 1. Lymphocyte subsets. Absolute counts of $CD3^+$, CD4/CD8 ratio and percentages of $CD45RA^+$ and $CD45RO^+$ cells in patients (n=13) at different time points after aSCT (mean levels ± SE; NA = not assessed). Normal values (median, minimal and maximal values) are from de Vries *et al*[15].

Lymphocyte subsets	3 months	6 months	14 months	normal values
$CD3^+$ ($x10^9$/L)	0.92 ± 0.22	0.79 ± 0.07	1.14 ± 0.19	1.5 (0.7-1.8)
CD4/CD8 ratio	0.30 ± 0.08	0.45 ± 0.03	0.59 ± 0.09	2.0 (1.2-2.7)
$CD45RA^+$ (% of $CD3^+$)	NA	30 ± 7.5	26 ± 4.6	60 (46-71)
$CD45RO^+$ (% of $CD3^+$)	NA	61 ± 7.6	65 ± 5.4	56 (48-71)

T-cell proliferation

Results of determination of in vitro T-cell proliferative capacity are shown in *Figure 1*. The proliferative response to stimulation with CD2 + CD28 mABs or with PHA increased between 6 and 15 months after aSCT. PHA-stimulated T-cell proliferation was significantly lower in patients compared to healthy controls at all time points, whereas CD2 + CD28-stimulated T-cell proliferation was not significant different in patients compared to healthy controls.

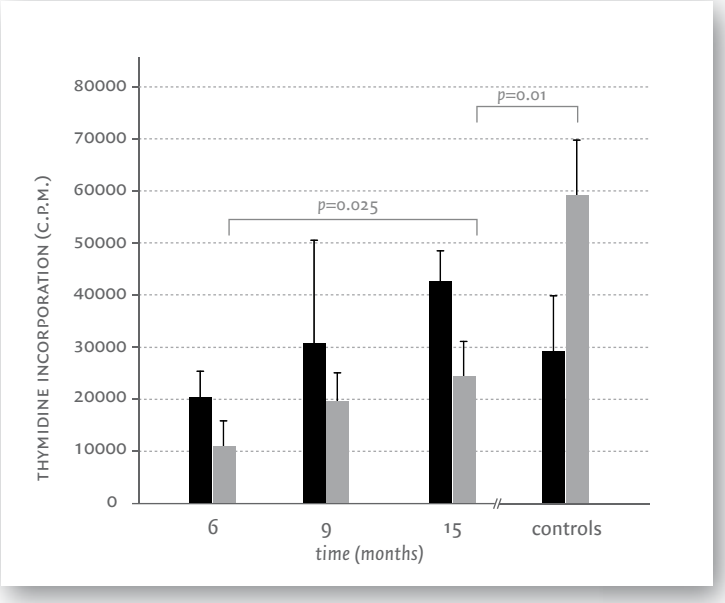

Figure 1. In vitro T-cell proliferation in patients at 6, 9 and 15 months after aSCT and in healthy controls: CD2 + CD28 (filled columns) and PHA stimulated cultures (hatched columns). Thymidine incorporation of cultures in medium was < 200 c.p.m. Data represent means + SEM from 13 patients and 5 healthy controls.

In vitro cytokine profile

Figure 2 shows levels of T helper-1 (Th1) cytokines (IL-2, IFN-γ and TNF-α) in supernatants of PBMC stimulated with CD2 + CD28 mABs and PHA at 6, 9 and 15 months post-transplantation. From 6 to 15 months after transplantation, an increase in IL-2, TNF-α and IFN-γ production was found (*p*-value: IL-2 0.036 and 0.051, TNF-α 0.036 and 0.021, and IFN-γ 0.05 and 0.021 for CD2 + CD28 and PHA, respectively). However, although levels of IL-2 were relatively high in patients compared to controls, levels of IFN-γ and TNF-α were strikingly lower in patients compared to healthy controls.

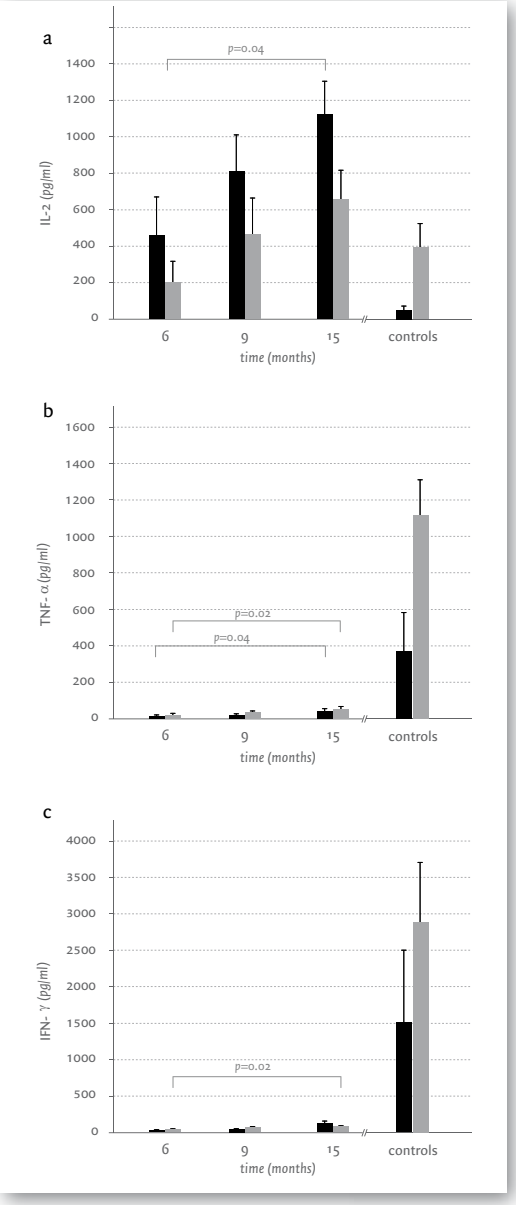

Figure 2. Mean levels (+ SEM) of Th1 cytokines IL-2 (A), TNF-α (B) and IFN-γ (C) in CD2 + CD28 (filled columns) or PHA (hatched columns) stimulated cultures set up at 6, 9 and 15 months after aSCT (n=13) and in controls (n=7). Cytokine concentrations in cultures in medium only, was < 0.4 pg/ml at all time points.

Levels of T helper-2 (Th2) cytokines (IL-5 and IL-13) are depicted in *Figure 3*. IL-5 and IL-13 levels increased from 6 to 15 months after transplantation (*p*-value: IL-5 0.036 and 0.038, IL-13 0.327 and 0.028 for CD2 + CD28 and PHA, respectively). Levels of the Th2 cytokine IL-4 were also determined, but were very low (mean levels 23.8 pg/ml and 2.6 pg/ml at 15 months after transplantation for CD2 + CD28 and PHA, respectively). At 15 months after transplantation, levels of IL-5 and IL-13 were significantly higher in patients than in healthy controls.

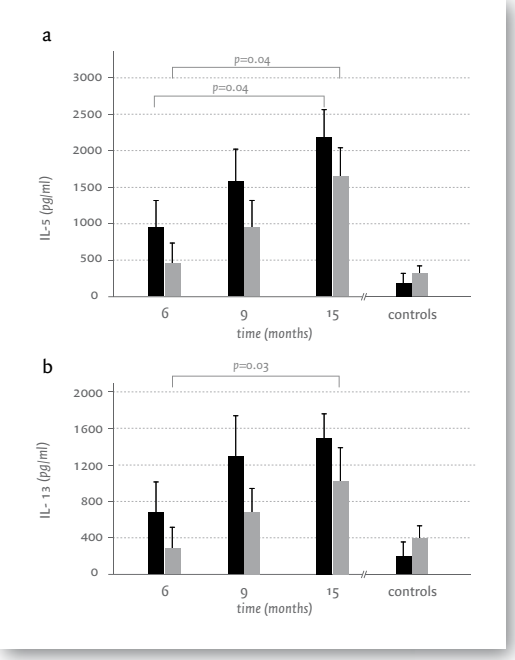

Figure 3. Mean levels (+ SEM) of Th2 cytokines IL-5 (A) and IL-13 (B) in CD2 + CD28 (filled columns) or PHA (hatched columns) stimulated cultures set up at 6, 9 and 15 months after aSCT (n=13) and in controls (n=7). Cytokine concentrations in cultures in medium only, was < 0.4 pg/ml at all time points.

Levels of the regulatory cytokine IL-10 are shown in *Figure 4*. IL-10 levels significantly increased from 6 to 15 months after transplantation (*p*-value: 0.036 and 0.028 for CD2 + CD28 and PHA, respectively).

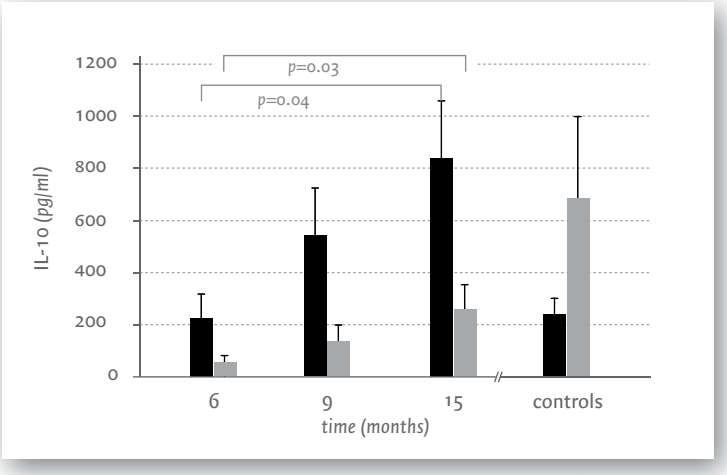

Figure 4. Mean levels (+ SEM) of IL-10 in CD2 + CD28 (filled columns) or PHA (hatched columns) stimulated cultures set up at 6, 9 and 15 months after aSCT (n=13) and in controls (n=7). Cytokine concentrations in cultures in medium only, was < 0.4 pg/ml at all time points.

Relatively low levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and the chemokine IL-8 were found (data not shown). This is probably related to the fact that supernatants were harvested after 6 days of culture, while these pro-inflammatory cytokines are predominantly secreted during the first 24-48 hours of cell culture.

In vitro stimulation of mononuclear cells with CD2 + CD28 mAbs or with PHA induces polyclonal T-cell activation. For extrapolation to the in vivo situation, the response pattern to antigen-specific T-cell activation is more relevant. Because all patients participated in our vaccination study consisting of 3 doses of vaccination with DTP and TT-conjugated *Hib* vaccine, we used TT to assess antigen-specific in vitro cytokine production. TT-stimulated T-cell proliferation significantly increased from 6 to 15 months after transplantation (*Table* 2; *p*-value 0.028 for 15 months versus 6 months). Also, TT-stimulated production of IL-2, TNF-α, IL-5, IL-13 and IL-10 significantly increased from 6 to 15 months after aSCT, i.e. after three vaccinations with DTP and TT-conjugated *Hib* vaccine (*Table* 2; *p*-values for 15 months versus 6 months were 0.017, 0.046, 0.028, 0.028 and 0.017 for IL-2, TNF-α, IL-5, IL-13 and IL-10, respectively). At 9 and 15 months after transplanta-

tion, the TT-induced Th2 cytokines IL-5 and IL-13 in our patients were at or above the levels induced in supernatants of PBMC cultures of healthy adults. Th1 cytokine induction was variable: activation with TT did induce significant amounts of IL-2 at 9 and 15 months after transplantation (i.e. after one and two doses of DTP and TT-conjugated *Hib* vaccine). Activation with TT however failed to induce IFN-γ, even at 15 months after transplantation (i.e. after three doses of DTP and TT-conjugated *Hib* vaccine).

Table 2. Tetanus toxoid stimulated cytokine production and T-cell proliferation. Mean TT-stimulated cytokine levels (pg/ml, ± SEM) and T-cell proliferation (thymidine incorporation in counts per minute (cpm), ± SEM) are given for cultures with PBMC obtained at 6, 9 and 15 months after aSCT (i.e. before and after 2 and 3 vaccinations, respectively; n=13) and in healthy controls (n=9). * *p*-values of cytokine levels of patients at 15 months versus controls are given.

	6 months	9 months	15 months	controls	*p*-value*
IL-2	1.1 ± 0.1	133.1 ± 62.4	348.7 ± 134.7	145.3 ± 70.9	0.241
IFN-γ	1.5 ± 0.1	1.8 ± 0.3	4.3 ± 2.8	1022.4 ± 542.8	0.042
TNF-α	0.7 ± 0.1	4.4 ± 1.8	13.7± 6.4	23.3 ± 9.1	0.384
IL-5	0.7 ± 0.1	206.2 ± 100.6	620.7 ± 253.6	83.3 ± 31.9	0.085
IL-13	0.65 ± 0.1	98.2 ± 43.0	291.5 ± 112.3	117.7 ± 63.4	0.234
IL-10	2.1 ± 0.5	135.0 ± 125.8	96.8 ± 39.0	511.2 ± 366.5	0.208
cpm	350 ± 70	5892 ± 1784	22546 ± 5578	5634 ± 791	0.003

Discussion

In this study, cellular immune function in autologous stem cell transplant recipients was investigated by measuring in vitro T-cell proliferation and the cytokine production profile. We found ongoing restoration of T-cell proliferation and cytokine production during the first 15 months after aSCT. Cellular immune function in terms of T-cell proliferation has been studied previously in autologous stem cell transplant recipients. T-cell proliferation responses were suppressed 3-12 months post-transplantation in patients with breast cancer or amyloidosis undergoing aSCT[16,17]. Studies regarding cytokine production after aSCT show conflicting results. Te Boekhorst *et al* compared T-lymphocyte reconstitution in T-cell depleted versus unselected aSCT in patients with multiple sclerosis

and haematological malignancies, respectively[18]. They found normal intracellular production of IFN-γ, IL-2 and TNF-α from 2 months post-aSCT onwards without significant differences between both groups. Guillaume *et al* studied cellular immune reconstitution in 66 patients receiving aSCT[19]. In 41 patients with a medium follow-up of 50 days post-transplantation, low levels of IL-3, IL-4, IFN-γ, IL-2 and IL-10 were found, while in 25 patients with a median follow-up of 654 days levels of these cytokines were comparable to healthy controls. Singh *et al* described low mRNA expression of Th1 cytokines IL-2, IFN-γ and TNF-α and normal mRNA expression of Th2 cytokine IL-4, 12 months after aSCT in patients with non-Hodgkin's lymphoma[20]. Levels of IL-10 were relatively high during the first 100 days after transplantation, but normalised thereafter. However, cellular function as measured by in vitro T-cell proliferation was depressed during the entire study period. They suggested that the initially high levels of IL-10 might explain the cellular immune dysfunction. IL-10 inhibits cytokine production and antigen stimulated T-cell proliferation by a direct negative effect on T lymphocytes as well as indirectly by down-regulation of class II MHC expression and thus antigen-presenting cells[21]. In our study, however, a strong suppressive effect of IL-10 on T-cell proliferation seems unlikely, since increases in IL-10 production were paralleled by significant increases in T-cell proliferation.

In the current study, a robust production of Th2 cytokines IL-5 and IL-13 was found 15 months after transplantation, while production of the Th1 cytokines IFN-γ and TNF-α remained low during the entire study period. Th2 cytokines are important for regulation of the humoral immune response[13,22]. In a previous report, we describe significant increases in quantitative and qualitative antibody responses to recall antigens following aSCT in this group of patients[9]. Our current results show superior recovery of antigen-stimulated production of the prototype Th2 cytokines IL-5 and IL-13 as compared to that of the Th1 cytokine IFN-γ. Schlenke *et al* also found impaired intracellular production of IFN-γ during the first 12 months after aSCT[23]. In mice, pre-treatment of donors with G-CSF resulted in decreased secretion of IFN-γ[24,25]. Sloand *et al* confirmed this decreased production of IFN-γ after administration of G-CSF in humans[26]. Although our patients did not receive G-CSF in the transplantation period, the stem cell mobilisation regimen (performed weeks to months before transplantation) included the administration of G-CSF. Thus, the low levels of IFN-γ may in part be attributed to the G-CSF pre-treatment.

A relatively high incidence of viral infections is observed for a prolonged period

after aSCT[17,18]. For instance, varicella zoster virus (VZV) infections are seen in approximately 50% of bone marrow transplant recipients, until 18 months after transplantation[27]. Th1 cytokines, and in particular IFN-γ, are important for development of cytotoxic effector T lymphocytes[13,28,29]. A deficient production of Th1 cytokines therefore might contribute to the occurrence of viral infections after aSCT. Because the currently available VZV vaccine is a live attenuated vaccine with potentially serious side effects in patients with impaired immune function, vaccination with VZV vaccine is not recommended in autologous stem cell transplant recipients during the first 2 years. Therefore, we vaccinated our patients with bacterial vaccines (DTP, *Hib* and pneumococcal vaccines) following the general guidelines[30,31]. Since our patients were vaccinated with TT-containing vaccines, we used TT as antigenic stimulus in our cell cultures. However, future studies concerning in vitro CMV- or VZV-stimulated cytokine production are necessary to elucidate a possible association between deficient production of Th1 cytokines and viral infections after aSCT.

In conclusion, our data show ongoing recovery of cellular immunity during the first 15 months after aSCT, which is reflected in an increase in in vitro T-cell proliferation and restoration of cytokine production. Production of Th1 cytokines IFN-γ and TNF-α, however, remain severely impaired during this period.

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CHAPTER 7

Summary and general discussion

Summary of the results

This thesis concerns the antibody responses to vaccination and immune function of patients with several forms of haematological diseases. Antibody responses in patients with chronic lymphocytic leukaemia (CLL) and in autologous stem cell transplant recipients were studied. In the autologous stem cell transplantation (aSCT) group, immune reconstitution after aSCT was analysed in detail during 15 months following transplantation.

In **chapter 1**, a general introduction is given. An overview of the increased infection rate and general as well as specific immune function in patients with CLL and autologous stem cell transplant recipients are presented. In patients with CLL, factors such as hypogammaglobulinaemia and deficient T helper cell function attribute to the risk of infections with encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenza* type b (*Hib*). In autologous stem cell transplant recipients, humoral and cellular immune functions are compromised during a prolonged period after transplantation, leading to a relatively high risk of infections in these patients.

In **chapter 2**, antibody responses to booster vaccination with influenza virus vaccine in patients with CLL are investigated. Response rates after the first vaccination were 5% and 15% for influenza A and influenza B, respectively. Booster vaccination resulted in an increase in response rates to 15% and 30% for influenza A and B, respectively. These response rates are low compared to response rates in healthy young adults or even in elderly. Thus, in patients with CLL, antibody responses to a single vaccination with influenza virus vaccine are severely impaired with only limited improvement after booster vaccination.

In **chapter 3**, antibody responses to vaccination with tetanus toxoid (TT) conjugated *Haemophilus influenzae* type b (*Hib*) vaccine and a non-conjugated 23-valent pneumococcal polysaccharide vaccine in CLL patients with and without ranitidine treatment are presented. In patients treated with ranitidine, post-vaccination anti-*Hib* and anti-TT geometric mean antibody titre (GMT) were 15.0 μg/ml and 1.03 μg/ml, respectively, which is higher as in CLL patients without ranitidine treatment (post-vaccination anti-*Hib* and anti-TT GMT of 4.07 μg/ml and 0.46 μg/ml, respectively). No beneficial effect of ranitidine on anti-pneumococ-

cal antibody titres was found. Thus, ranitidine treatment can enhance T-cell dependent antibody responses but not T-cell independent antibody responses to vaccination.

In **chapter 4**, the immune response to vaccination and development of immune function after aSCT in the Vaccination after Autologous Stem cell Transplantation (VAST) trial were studied. Patients were vaccinated with *Hib* vaccine at 6, 8 and 14 months after aSCT. After three vaccinations, the response rate (defined as a $\geq$ 4-fold increase in antibody titre) was 94%. In addition to quantitative antibody responses, we also studied functionality of anti-*Hib* antibodies. To that end, antibody avidity was measured by a sodium thiocyanate elution method, which revealed significant increases in antibody avidity after three vaccinations with conjugated *Hib* vaccine. Furthermore, we developed a quantitative assay for analysis of the phagocytosis of antibody coated (FITC labelled) *Hib* bacteria by granulocytes. The level of phagocytosis can be expressed as the mean FITC fluorescence intensity (MFI) of granulocytes. Mean MFI increased significantly after two and three vaccinations with conjugated *Hib* vaccine. In general, an increase in anti-*Hib* antibody levels was accompanied by an increase in antibody avidity and phagocytosis-inducing capacity. In conclusion, the vaccination schedule resulted in adequate antibody response rates and functional maturation of anti-*Hib* antibodies. After two vaccinations with *Hib* vaccine, antibody titres and antibody avidity were comparable to healthy controls that were vaccinated with a single dose. However, phagocytosis-inducing capacity remained suboptimal until the third vaccination. Therefore, we advise three vaccinations with *Hib* vaccine in autologous stem cell transplant recipients.

In **chapter 5**, in order to evaluate the antibody response to vaccination in the context of the cellular immune reconstitution, we determined peripheral blood lymphocyte subsets and function in patients included in the VAST trial. $CD3^+$ cells (T lymphocytes), $CD16^+CD56^+$ cells (natural killer cells), $CD19^+$ cells (B lymphocytes), $CD4^+$ cells (helper T lymphocytes), $CD8^+$ cells (cytotoxic T lymphocytes), $CD45RA^+$ cells (naive T lymphocytes) and $CD45RO^+$ cells (mature T lymphocytes) were determined by flowcytometry at regular intervals. At 3 months after aSCT, levels of $CD3^+$ and $CD16^+CD56^+$ cells were in the normal range. Levels of $CD19^+$ cells were in the lower normal range at 3 months after transplantation and significantly improved during the months thereafter. Although increasing

during the study period, a persistently low CD4/CD8 ratio was found. Moreover, while levels of $CD45RO^+$ cells were in the normal range at 6 months post-transplantation, levels of $CD45RA^+$ cells were persistently low at 6 and 14 months after transplantation. Thus, natural killer cells and total number of T lymphocytes normalise early after aSCT, whereas B lymphocytes and subsets of T lymphocytes (especially $CD4^+$ and $CD45RA^+$ cells) remain low during a longer period. We analysed the correlation between lymphocyte subsets and antibody responses and found a relation between $CD19^+$ cell counts and anti-*Hib* antibody responses. All patients with $CD19^+$ cell counts of $\geq 0.20 \times 10^9$/L before vaccination showed an adequate response (defined as a ≥ 4-fold increase in antibody titre) to a single vaccination with *Hib*-vaccine, while all patients with $CD19^+$ cell counts of $\leq 0.10 \times 10^9$/L required at least two vaccinations before sufficient antibody levels were obtained. No relation was found between kinetics or magnitude of the antibody response and T-cell numbers of subsets (CD4/CD8 ratio, $CD45RA^+$, $CD45RO^+$ cells). Thus, a minimum threshold level of $\geq 0.20 \times 10^9$/L $CD19^+$ cells appears to be required for adequate responses to vaccination.

In **chapter 6**, the functional reconstitution of cellular immunity of the patients participating in the VAST-study is assessed. We determined in vitro T-cell proliferation and cytokine production at 6, 9 and 15 months after aSCT. Peripheral blood mononuclear cells (PBMC) were stimulated with a combination of CD2 and CD28 monoclonal antibodies, with PHA or with tetanus toxoid as recall antigen. T-cell proliferation significantly increased from 6 to 15 months after transplantation. Levels of T helper 1 (Th1) cytokines IL-2, IFN-γ and TNF-α, T helper 2 (Th2) cytokines IL-5 and IL-13 and the regulatory cytokine IL-10 all increased from 6 to 15 months after transplantation. However, levels of IFN-γ and TNF-α were strikingly lower in patients compared to healthy controls, whereas at 15 months post-transplantation, levels of IL-5 and IL-13 were significantly higher in post-transplantation patients compared to controls. Th2 cytokines are important regulators of the humoral immune response against bacterial infections, whereas Th1 cytokines have an important role in the cellular immune pathway with anti-viral immune activity. The adequate production of Th2 cytokines fits with the observed adequate in vivo antibody responses after vaccination as described in chapter 4, whereas the subnormal production of Th1 cytokines might contribute to the relatively high occurrence of viral infections after aSCT.

General discussion

The aim of this thesis was to analyse immune function and reconstitution and improvement of antibody responses by vaccination in patients with chronic lymphocytic leukaemia (CLL) and autologous stem cell transplant recipients. Patients with haematological malignancies frequently have deficient immune function caused by factors related to the disease itself and because of immune suppressive therapy. The monoclonal proliferation of the malignant cell line, for instance lymphocytes in patients with CLL, can ultimately lead to elimination of normal white blood cells with negative effects on immune function. Additionally, chemotherapy suppresses immune function, leading to an increased infection risk in these patients[1]. Moreover, high dose chemotherapy with autologous stem cell transplantation (aSCT) is a frequently used regimen in patients with haematological malignancies. In autologous stem cell transplant recipients, after recovery from neutropenia, immune function may remain impaired for a prolonged period[2]. With respect to studies of immune function, it has to be kept in mind that in patients with CLL, the immune status deteriorates with advancement of the disease whereas in autologous stem cell transplant recipients, the immune status shows a gradual improvement after transplantation. The results of this thesis in both patient groups will be discussed in the context of infection prevention in these patients.

Patients with CLL

Infection prevention in patients with CLL

As described in detail in the introduction of this thesis, patients with CLL are prone to infections with serious morbidity and mortality[1]. The incidence of community-acquired pneumonia (CAP) varies between 1-9/10^3 per patient year (ppy) in the general adult population with the higher incidences at the extreme ages of life[3,4]. We assessed the incidence of serious pneumonia (defined as pneumonia confirmed by chest film and/or clinical suspicion of pneumonia with positive bacterial pathogen cultures of blood or sputum, for which admittance to the hospital was necessary) in 25 patients of our CLL cohort. In these patients with a mean follow up of 7.3 years (range 1.5-13 years, total of 185 patient years), the incidence of CAP was 50/10^3 ppy, indicating a highly increased risk for developing CAP in patients with CLL. Multiple factors, including defects in humoral[5,6] and

cellular immunity[7,8] contribute to this susceptibility. For example, CLL patients may have hypogammaglobulinaemia leading to a higher risk of recurrent bacterial infections as compared to patients without hypogammaglobulinaemia[9].

Several strategies, such as intravenous immunoglobulin (IVIG) and prophylactic antibiotics, are used to reduce infections and infection-related complications. IVIG has been shown to reduce moderately severe infections but not minor or severe infections in CLL patients[10-12]. Based on these results, a consensus statement concerning the use of IVIG in patients with various diseases was published in 1990. IVIG was advised for patients with CLL and hypogammaglobulinaemia to prevent serious bacterial infections[13]. However, although some beneficial effects of IVIG in reducing the risk of infections were seen, no effects on overall survival were found[14]. It can be questioned whether the use of IVIG in CLL patients is a cost-effective approach and cost-effectiveness analyses are warranted[15]. Apart from the hypogammaglobulinaemia, there may be additional defects in cellular immunity in patients with CLL (see below), which cannot be corrected by IVIG replacement therapy.

An alternative strategy for infection prevention is enhancement of adaptive immunity by vaccination. Most vaccination studies in patients with CLL concern vaccination with influenza virus, *Hib* and *Streptococcus pneumoniae* vaccine. Vaccination with influenza virus vaccine in patients with CLL results in disappointingly low response rates[16,17]. In order to improve antibody responses to influenza virus vaccine, we used a booster vaccination schedule that only resulted in a limited improvement of antibody responses. It should be added that host defence against influenza virus primarily depends on cellular immunity[18]. We have not tested the cytotoxic T-lymphocyte response to influenza virus in our patient group. However, the modest antibody response to influenza virus vaccination in CLL patients warrants the search for alternative strategies to reduce morbidity and mortality by influenza virus infection. Intranasally administred live attenuated influenza virus vaccine shows improved efficacy in toddlers compared to trivalent inactivated influenza vaccine, with limited adverse effects[19]. The use of this vaccine might be considered in patients with CLL, although the efficacy and safety profile in this particular group of patients are not known yet.
Apart from vaccination, anti-viral drugs like amantadine and rimantadine have been used. These drugs have high oral bioavailability and low costs but restricted

efficacy against influenza virus type A (not type B). Additionally, development of resistance limits their use. The relatively new anti-viral drugs zanamivir and oseltamivir inhibit the neuraminidase enzyme, an essential enzyme for influenza virus type A and B. These drugs have been proven to reduce the risk of influenza virus infection related complications in high-risk groups (mostly patients with pulmonary diseases) when administered early after onset of symptoms[20-22]. Neuraminidase inhibitors have also been used successfully as prophylaxis after exposure to influenza virus in elderly[23]. In a small, non-randomised study concerning the therapeutic use of zanamivir in allogeneic stem cell transplant recipients, no adverse effects were seen[24]. In my opinion, neuraminidase inhibitors should be advised in high-risk patients, although larger, randomised studies are necessary to establish the efficacy of neuraminidase inhibitors in patients with haematological malignancies.

In addition to viral infections such as influenza virus infection, common nasal commensals like *Streptococcus pneumoniae* are important causes of infection in CLL patients[9,25]. *Hib* also used to be a pathogen but due to herd-immunity effects after the introduction of *Hib* in the national infant vaccination programme, is much less frequent nowadays[26]. Nevertheless, vaccination with conjugate *Hib* vaccine is warranted for optimal protection against potential very serious invasive disease. Thus, vaccination with *Streptococcus pneumoniae* and *Hib* vaccine in patients with CLL seems a good preventive strategy. However, as for vaccination with influenza virus vaccine, responses to vaccination with *Hib* and pneumococcal vaccines are often very low in these patients[27,28]. In accordance with earlier studies[29,30], we found improved T-cell dependent antibody responses to vaccination during ranitidine treatment in patients with CLL. No beneficial effect of ranitidine treatment on T-cell independent antibody responses to non-conjugated polysaccharide vaccine was found. However, the effect of ranitidine treatment on antibody responses to conjugated pneumococcal vaccine in patients with CLL was not assessed. A vaccination schedule consisting of conjugated pneumococcal vaccine in addition to conjugated *Hib* vaccine during ranitidine treatment could be further evaluated. Recently, the use of ranitidine has been associated with a higher incidence of CAP[31,32]. Therefore, the potential adverse effects of ranitidine treatment should be balanced by the potential positive effects of ranitidine on antibody responses. Booster vaccination in this group of patients might be a more efficient option with less adverse effects.

The prophylactic use of antibiotics offering protection against encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (*Hib*) has been recommended in CLL patients with recurrent upper airway infections[33]. However, no randomised controlled trials confirming a beneficial effect of prophylactic antibiotics have been published. A disadvantage of the extensive use of antibiotics is the potential emergence of resistant microorganisms. Alternatively, antibiotics can be used in a pre-emptive setting at the first sign of an infection.

The risk for infections in patients with CLL is related to disease stage, previous courses of chemotherapy and hypogammaglobulinaemia[1]. Also, vaccination responses are better in patients with less advanced disease stage and normal gammaglobulin levels[1,28]. However, patients with adequate antibody responses to vaccination in early stage CLL, might lose the acquired antigen-specific memory when the disease progresses. In progressive disease, proliferation of malignant B cells results in expulsion of benign cells. If memory B cells are replaced by malignant B cells, the memory to the recall antigens therefore can be lost. It is, however, not clear which benign cells are replaced by the malignant clone. Should naive B cells but not memory B cells be eliminated, the acquired memory will be retained. Further research concerning this issue, by longitudinal analysis of $CD27^+$ memory B lymphocytes is warranted.

Recently, new prognostic factors have been identified for patients with CLL. IgVH mutation status, CD38 and ZAP-70 have been proven to correlate with disease progression in CLL patients[34,35]. Francis *et al* studied infections in patients with CLL in the context of these new prognostic factors. Cytogenetic profile, CD38 expression and IgVH mutations status indeed were associated with infection-related mortality[36]. Studying the correlations between antibody responses to vaccination and these prognostic factors might provide additional factors predicting favourable responses to vaccination. None of these risk factors were analysed in our patient cohort and, unfortunately, no biological material was available for retrospective analysis. Moreover, new treatment modalities for patients with CLL emerge, such as the use of the strongly immune suppressive drug fludarabine combined with cyclophosphamide as first line therapy in patients with adverse prognostic factors. Additionally, monoclonal antibodies (for example rituximab and alemtuzumab) and stem cell transplantation are increasingly used in these

patients. The impact of these treatments on immune function and infection risk should be carefully assessed in the following years.

In conclusion, infection prevention by vaccination should be performed in early stages of CLL and preferably before the first course of chemotherapy. However, given the fact that the immune status of a CLL patient worsens over time, B-cell memory might not last, or in other words, the patient might not remain protected over a prolonged period.

What are the implications of this research for general practice?

Based on our data, existing literature and the official guidelines, we suggest the following:

» Patients with CLL should be immunised annually with a single dose of influenza virus vaccine. Additionally, we recommend a booster vaccination 3-6 weeks after the first vaccination. Although antibody responses are often low, serious adverse effects of influenza virus vaccination are extremely rare. Thus, a small proportion of the patients will benefit from annually vaccination with influenza virus vaccine, whereas the discomfort for non-responding patients is acceptable.
» The neuraminidase enzyme inhibitors zanamivir and oseltamivir have been proven to be successful in a pre-emptive as well as a prophylactic setting in high-risk groups. Therefore, we recommend the use of these anti-viral drugs in patients with CLL. However, additional studies are necessary to confirm a beneficial effect of neuraminidase enzyme inhibitors in this group of patients.
» In CLL patients with hypogammaglobulinaemia and documented recurrent infections, intravenous immunoglobulin therapy according to the general guidelines can be considered. However, since the use of IVIG in this group of patients might not be a cost-effective approach, the effectivity of IVIG should be individually assessed at regular intervals.
» Vaccination responses are better in CLL patients with less advanced disease stage, normal gammaglobulin levels and no or only limited courses of chemotherapy. Therefore, vaccination of CLL patients with *Streptococcus pneumoniae* and *Hib* vaccine should preferably be performed in an early disease stage and before the first course of chemotherapy.
» Ranitidine treatment improves T-cell dependent antibody responses but has

no beneficial effects on antibody responses to non-conjugated pneumococcal vaccine. Because of the higher incidence of CAP associated with long-term use of ranitidine, we do not recommend the addition of ranitidine to a vaccination schedule with *Hib* and *Streptococcus pneumoniae* vaccine.

Autologous stem cell transplant recipients

Infection prevention in autologous stem cell transplant recipients

The conditioning regimen for autologous stem cell transplantation (aSCT) leads to a significant impairment of immune function for a prolonged period after transplantation. Like in CLL, infections caused by encapsulated bacteria such as *Streptococcus pneumoniae* and previously *Hib*, are major sources of morbidity in aSCT[37,38]. Vaccination of stem cell transplant recipients with, amongst others, pneumococcal vaccine and *Hib*-vaccine has been recommended by institutions such as the Centers for Disease Control and Prevention, the American Society of Hematology and the European Group for Blood and Marrow Transplantation[39-41]. Most currently used revaccination schedules start at 12 months after aSCT, although more recent guidelines advice the start of revaccination at 6 months after transplantation (see *Table* 1 and *Table* 2), which should reduce the duration of unprotected state.

Table 1. Summary of the vaccination recommendations by the Centers for Disease Control and Prevention (CDC), Infectious Diseases Society of America (IDSA) and American Society for Blood and Marrow Transplantation (ASBMT)[40]: number of doses and starting time after transplantation. Recommendations concerning the use of a conjugated pneumococcal vaccine were not given.

		Guidelines CDC, IDSA and ASBMT 2001	
		no. of doses	first time after a SCT
Hib conjugate		3	12 months
S. *pneumoniae*,	conjugated (7-valent)	n.a.	n.a.
	non-conjugated (23-valent)	2	12 months, 2nd dose at 24 months
DT(P)		3	12 months

Table 2. Summary of the EBMT vaccination recommendations[41]: number of doses and starting time after transplantation.

		Guidelines EBMT 2005	
		no. of doses	first time after a SCT
Hib conjugate		3	6 months
S. *pneumoniae*,	conjugated (7-valent)	3	6-12 months.
	non-conjugated (23-valent)	1	12 months
DTP		3	6-12 months

Vaccines that are administered in a very immature immune system may not result in adequate antibody responses. Moreover, there is a potential risk of induction of immunological tolerance although this never has been shown for conjugate vaccines in infants. Kähty *et al* studied anti-*Hib* antibody responses after vaccination in young children[42]. In all instances, anti-*Hib* antibodies were higher in the vaccinated children compared to controls. Moreover, all children who did not

respond to the first vaccination with *Hib* polysaccharide vaccine (because of their young age), showed adequate antibody responses after a booster vaccination. Thus, no signs of development of tolerance were found.
In our population of autologous stem cell transplantation recipients, all except one patient showed adequate anti-*Hib* antibody responses after three vaccinations with *Hib* vaccine. The single patient who did not respond to the vaccinations had undetectable levels of B lymphocytes during the entire vaccination period, which explains the lack of response. In the patients with protective anti-*Hib* antibody levels after a single vaccination (defined as ≥ 1.0 µg/ml), the geometric mean anti-*Hib* antibody titre after the full vaccination schedule was 102 µg/ml (95% confidence interval 39.8-263.3). In patients with anti-*Hib* antibody levels below the protective threshold of 1.0 µg/ml after the first vaccination with *Hib* vaccine, the geometric mean anti-*Hib* antibody titre after three vaccinations was 23 µg/ml (95% confidence interval 2.5-208.4). This difference in antibody titres between responders and non-responders to the first vaccination with *Hib* vaccine was not statistically significant. Therefore, no evidence for induction of tolerance by a vaccination schedule starting 6 months after aSCT (*Table 3*) was found.

Table 3. Vaccination schedule according to the VAST-trial

Time after transplantation		
6 months	8 months	14 months
DTP-vaccine conjugated *Hib*-vaccine 7-valent conjugated pneumococcal vaccine	DTP-vaccine *Hib*-vaccine 7-valent conjugated pneumococcal vaccine	DTP-vaccine *Hib*-vaccine 23-valent non-conjugated polysaccharide pneumococcal vaccine

Although vaccination too early after transplantation may not be efficient, unnecessary delay of vaccination puts patients at prolonged risk for infections. For conjugated *Hib*-vaccine, our data show high efficacy of revaccination starting at 6 months after transplantation without major side effects. This was not surprisingly, since carrier-conjugated vaccines have been proved to be immunogenic in immature immune systems. However, for the pneumococcal vaccine this does not hold true because our data show insufficient response rates after two doses of conjugated pneumococcal vaccine in autologous stem cell transplant recipients

8 months after BMT. The immaturity of the immune system during infancy may however not be comparable with the immaturity of the immune system in adults after aSCT. Possibly, the antibody response to conjugated pneumococcal vaccine is different in dependency from B- and T-cell recovery than the anti-*Hib* antibody response. Thus, other strategies such as additional doses of conjugate vaccine are needed to enhance anti-pneumococcal antibody responses. Pneumococci are the dominant cause for CAP, bacteraemia and meningitis. Optimal clinical care of this group of patients therefore includes adequate preventive measures against these infections.

Currently, the only available conjugated pneumococcal vaccine is the heptavalent vaccine, consisting of capsular polysaccharides of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. In children, invasive pneumococcal disease (IPD) is most often caused by these pneumococcal serotypes. In adults, however, IPD is frequently caused by pneumococcal serotypes 3, 7, 8 and 12 in addition to serotypes 1, 4, 9V and 14[43,44]. Thus, the heptavalent conjugated pneumococcal vaccine is not optimal in protecting adults from IPD, with a theoretical coverage of less than 50% of pneumococcal serotypes. Therefore, the development of 11-valent or even 13-valent conjugate pneumococcal vaccines is of utmost importance for adult patients, extending the serotype coverage.

Another strategy to enhance immune responses to vaccinations is the use of adjuvants. Adjuvants are structures that are not immunogenic by itself but augment vaccine responses by yet unresolved mechanisms that may involve enhanced antigen presentation (and activation of the innate immune system) and cytokine production[45]. A considerable number of potential adjuvants is available, including mineral salts, polymers and synthetic constructs[46]. CpG-containing oligodeoxynucleotide is a synthetic adjuvant with proven beneficial effects on responses to DTP and conjugated *Hib* vaccine in mice[47,48]. For use in humans, only aluminium salts are allowed at this moment.

Vaccination of patients prior to high dose chemotherapy and aSCT has also been performed. Molrine *et al* immunised patients with pneumococcal polysaccharide vaccine before bone marrow collection followed by two doses of pneumococcal polysaccharide vaccine at 12 and 24 months after autologous bone marrow transplantation[49]. This regimen did not result in higher anti-pneumococcal antibodies

compared to the control group, consisting of aSCT patients who were vaccinated with two doses of pneumococcal polysaccharide vaccine at 12 and 24 months after transplantation only. Almost ten years later, after the heptavalent conjugate pneumococcal vaccine became available, the same study group found beneficial effects of pre-transplantation vaccination in aSCT patients. Patients were vaccinated with the heptavalent pneumococcal conjugate vaccine before stem cell harvest and at 3, 6 and 12 months after aSCT whereas controls were vaccinated with the heptavalent pneumococcal conjugate vaccine at 3, 6 and 12 months after transplantation only. This resulted in higher anti-pneumococcal antibodies in patients compared to the control group, especially during the first 6 months after transplantation. At 13 months after transplantation, over 60% of all patients had protective antibody levels after the third vaccine dose regardless of pre-transplantation vaccination[50].

Although many studies focus on the prevention of bacterial infections after aSCT, viral infections can also lead to serious, and sometimes life-threatening complications in aSCT patients. For instance, varicella zoster virus (VZV) infection is seen in 20-50% of patients, mainly during the first 18 months after transplantation[51,52]. Treatment with acyclovir is the treatment of choice but infections due to drug-resistant VZV can become a clinically important problem. The currently available VZV vaccine is a live attenuated vaccine with potential serious adverse effects. Therefore, vaccination of seronegative aSCT patients should be performed either before or ≥ 2 years after transplantation[41]. Hata *et al* studied the use of a new, heat-inactivated VZV vaccine in VZV-seropositive patients with lymphoma undergoing aSCT[52]. One dose of the heat-inactivated vaccine before transplantation and three doses after transplantation resulted in a decreased incidence of zoster infections compared to non-immunised patients. The beneficial effects of this vaccine should be confirmed in other populations undergoing aSCT. At this moment, the heat-inactivated VZV vaccine is licensed in the USA but not routinely available. In allogeneic stem cell transplant recipients, anti-viral prophylaxis with acyclovir has been proven to be efficient in preventing VZV infections during the first year after transplantation[53]. Data concerning anti-viral prophylaxis in adult autologous stem cell transplant recipients are scarce. Sempere *et al* found no VZV infections in patients receiving prophylaxis with acyclovir[54]. Within one month after cessation of prophylaxis, however, 24% of patients developed localised herpes zoster. It is not clear whether anti-viral prophylaxis with acyclovir

only delays VZV infection in aSCT patients, or also has a beneficial effect on the seriousness of the infections.

Finally, vaccination of family members and hospital personnel might reduce the risk of exposure to infectious diseases in the patient. Many hospitals in The Netherlands already offer influenza virus vaccination to their employees. Vaccination of family members with influenza virus vaccine is not a generally accepted strategy in The Netherlands. Considering the seasonal exposure of patients to influenza infections, data regarding cost-effectiveness of especially influenza virus immunisation of family and hospital staff are needed.

What are the implications of this research for general practice?

» Revaccination of aSCT patients can safely be started at 6 months after transplantation; pending newer strategies to improve pneumococcal vaccine responses (such as the use of newer adjuvants), the vaccination schedule described in this thesis can be followed *(Table 3)*.
» The administration of the currently available live attenuated VZV vaccine to a recipient with an impaired immune system has potential serious side effects. Since an effective and safe alternative with prophylactic anti-viral drugs exists, the live varicella zoster vaccine should not be used in autologous stem cell transplant recipients during the first years after transplantation. Randomised controlled trials evaluating the safety and efficacy of the new heat-inactivated varicella zoster vaccine in haematological patients are needed.
» Because of the high seasonal exposure to influenza virus and the potentially life threatening complications of influenza virus infections in aSCT patients, vaccination of hospital staff and family members of these patients with influenza virus vaccine should be vigorously encouraged.

Long-term management after aSCT: is revaccination indicated?

Patients with high-risk lymphoma receiving high-dose chemotherapy and aSCT have an overall survival (OS) rate of 20-50% at four years after aSCT[55]. In patients with multiple myeloma, high-dose chemotherapy followed by aSCT results in improved overall survival rates compared to conventional chemotherapy (5 years OS: 52% versus 12%)[56]. Considering these improving survival rates, questions

regarding long-term management of autologous stem cell transplant recipients, including infection prevention, emerge.

In vaccinated asplenic children, anti-pneumococcal antibodies decline during years after vaccination[57]. Children vaccinated with 3 doses of conjugated *Hib* vaccine show a decline in anti-*Hib* antibody levels during the first year after immunisation[58]. It can be expected that anti-pneumococcal and anti-*Hib* antibodies also will decline in aSCT-patients in the period after completing a revaccination schedule consisting of multiple vaccinations with conjugated vaccines. Revaccination with pneumococcal vaccine of splenectomised children 5 years after primary vaccination has been reported to result in adequate antibody responses[59]. In view of these data, revaccination of aSCT patients at for instance 5 years after completing the revaccination schedule seems reasonable. Studies on the long-term incidence of infections after aSCT are, however, limited. Chen *et al* found a cumulative incidence of a first pneumonia episode (peri-transplantation pneumonia excluded) of 15% at 2 years, increasing to 18% at 4 years after aSCT[60]. In an EBMT survey concerning invasive pulmonary infections (IPI) after stem cell transplantation, 9 (18%) of 51 episodes of IPI occurred > 4 years after transplantation in a population with a variable vaccination status[61]. Thus, the available clinical data show an increased incidence of (pneumococcal) pneumonia in the period > 2 years post aSCT, indicating the need for long-term vaccinations strategies. Additional data evaluating the incidence of infections years after aSCT and cost-effectiveness studies analysing pneumococcal booster vaccination at for instance 5 years after aSCT are needed.

New therapeutic modalities and potential complications

Although high-dose chemotherapy followed by aSCT has improved response rates compared to conventional chemotherapy alone, eventually all patients with multiple myeloma will relapse. Therefore, additional and/or alternative therapeutic regimens are being developed. Currently, bortezomib and thalidomide are being evaluated in patients with relapsed multiple myeloma. Bortezomib is an inhibitor of the 26S proteasome and directly induces apoptosis of tumour cells by various mechanisms, such as inhibition of activation of NF-κB in tumour cells and in tumour microenvironment[62,63]. Recent data also indicate that bortezomib inhibits in vitro cytokine production by T cells[64]. The authors speculate

that this might result in impaired anti-bacterial and anti-viral immune response. Although the reported incidence of infectious complications during treatment with bortezomib is relatively low[65,66], the use of prophylactic anti-pneumococcal antibiotics, antifungal medication and anti-viral drugs is recommended in most study protocols evaluating bortezomib. The incidence of serious bacterial infections during treatment with thalidomide (with or without dexamethasone) is also low[67,68]. No data exist regarding the influence of bortezomib or thalidomide on the outcome of (re)vaccination with pneumococcal vaccine. Thus, further studies regarding infections and vaccination responses in aSCT patients with relapsed multiple myeloma being treated by bortezomib and/or thalidomide are needed to refine guidelines concerning infection prevention in these patients.

In patients with diffuse large cell lymphoma treated with chemotherapy, maintenance therapy with the monoclonal anti-CD20 antibody rituximab results in higher failure free survival rates[69]. If proven to be beneficial, treatment with rituximab during aSCT and/or as maintenance therapy after aSCT may become standard care in patients with high-risk lymphoma within several years from now. The CD20 molecule is expressed on all B lymphocytes and therefore treatment with rituximab results in B-cell depletion that persists for 6-9 months after cessation of therapy[70]. Van der Kolk *et al* studied antibody responses to TT and poliomyelitis vaccination in patients with relapsed, low-grade lymphoma before and during treatment with rituximab[71]. Humoral immune responses to these recall antigens were significantly decreased during rituximab treatment compared to responses before rituximab treatment. These adverse effects of rituximab on vaccination responses should be kept in mind while deciding on pneumococcal or *Hib* booster vaccination several years after aSCT in patients receiving rituximab.

Thus, the risk of infections several years after aSCT is unclear and data regarding the efficacy of long-term revaccination of aSCT patients are lacking. We strongly advise the revaccination of autologous stem cell transplant recipients during the first year after transplantation following a vaccination schedule as described in this thesis. Moreover, the impact of the newer treatment strategies including bortezomib, thalidomide and rituximab on infection risk and response to vaccination after aSCT should be further elucidated.

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Samenvatting voor niet-ingewijden

'De meest belangrijke ideeën van de wetenschap zijn in de grond eenvoudig en kunnen als regel worden meegedeeld in een taal die voor iedereen begrijpelijk is.' - A. Einstein

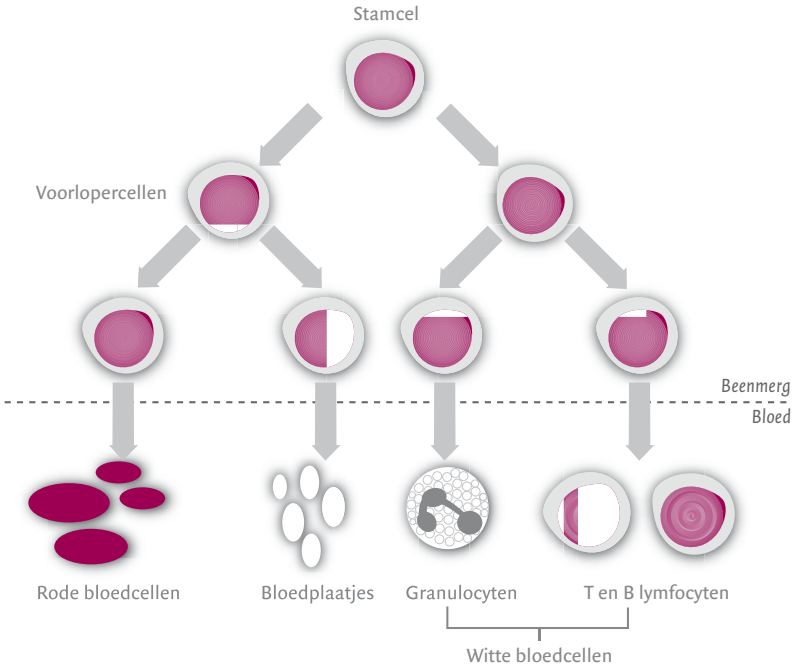
Aan niet-ingewijden uitleggen wat het vakgebied hematologie inhoudt, is niet eenvoudig. Om in "lekentaal" de inhoud van dit proefschrift te bespreken is helemaal een bijzondere uitdaging. Daarom wordt eerst een korte inleiding over de hematologie gegeven, gevolgd door een beknopte samenvatting van de resultaten uit dit proefschrift en de aanbevelingen voor verder onderzoek.

Inleiding

Hematologie is de leer van het bloed en de bloedziekten. Bloed bestaat uit vloeistof (bloedplasma) en bloedcellen. Hieronder volgt eerst een uitleg over de verschillende soorten bloedcellen. Omdat dit proefschrift gaat over afweer bij mensen met ziekten van het bloed (hematologische aandoeningen), wordt kort ingegaan op afweercellen en afweerreacties. Daarna worden de voor dit proefschrift belangrijke hematologische ziektebeelden en de verschillende vormen van behandeling uitgelegd.

Bloedcellen en afweer

Er bestaan 3 verschillende vormen van bloedcellen: bloedplaatjes, rode bloedcellen en witte bloedcellen, ieder met een eigen functie. Bloedplaatjes zijn belangrijk voor de bloedstolling, rode bloedcellen zorgen voor het transport van zuurstof door het lichaam en witte bloedcellen zijn belangrijk voor de afweer tegen infecties. De aanmaak van al deze 3 vormen van bloedcellen vindt plaats in het beenmerg. Beenmerg bevindt zich in de holle ruimtes van bepaalde botten (zoals bekken, borstbeen, schedel). In het beenmerg rijpt de onrijpe hematopoietische ("bloedvormende") stamcel via verschillende voorlopercellen uit tot bloedplaatjes, rode bloedcellen en witte bloedcellen (zie *Figuur 1*). De stamcel en voorlopercellen bevinden zich in het beenmerg, terwijl de rijpe cellen voornamelijk in het bloed aanwezig zijn. Voor dit proefschrift zijn vooral de witte bloedcellen van belang.

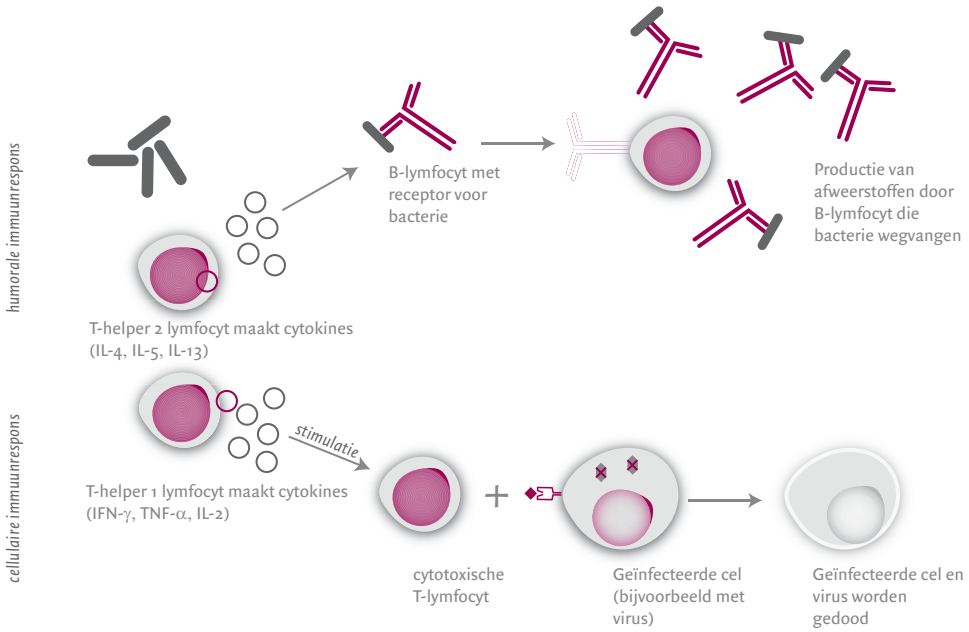


Figuur 1. Schematisch overzicht van bloedvorming.

Er zijn verschillende soorten witte bloedcellen, zoals granulocyten en lymfocyten. De lymfocyten zijn verder onder te verdelen in T-lymfocyten (waarbij de T staat voor thymus, de zwezerik) die zich in de thymus ontwikkelen tot rijpe lymfocyten, en de B-lymfocyten (de B staat voor Bursa van Fabricius, het orgaan waarin bij vogels de B-lymfocyten uitrijpen). De T-lymfocyten zijn onder te verdelen in T-helper cellen en zogenaamde cytotoxische T-cellen. Cytotoxische T-lymfocyten zijn T-cellen die geïnfecteerde cellen in het lichaam direct doden (*Figuur 2*). Deze afweerreactie wordt ook wel de cellulaire immuunrespons genoemd. Wanneer een B-lymfocyt in contact komt met een bacterie dan herkent een receptor op de B-lymfocyt een onderdeel van de bacterie (het antigen) waarna de B-cel afweerstoffen (immuunglobulinen) gaat produceren (*Figuur 2*). De afweerreactie waarbij afweerstoffen worden gemaakt, wordt de humorale immuunrespons genoemd. Om deze productie in gang te zetten is vaak hulp nodig van T-helper cellen. Vervolgens ontwikkelen de B-cellen zich tot plasmacellen die terugkeren

in het beenmerg. Tijdens de afweerrespons ontstaan ook B-geheugencellen die jarenlang in het lichaam aanwezig blijven. Wanneer dezelfde bacterie of virus een tweede keer het lichaam binnen dringt, zorgen de geheugencellen voor een snelle productie van grote hoeveelheden immuunglobulinen waardoor de infectie efficiënter bestreden kan worden, zodanig efficiënt dat hij ongemerkt voorbijgaat.

Iedere afweerreactie moet nauwkeurig worden gereguleerd: de reactie mag niet te zwak zijn, maar ook niet te sterk, moet op tijd starten en ook weer op tijd stoppen, enzovoort. Deze regulatie wordt verzorgd door cytokines, kleine eiwitten die de communicatie tussen verschillende cellen tijdens afweerreacties verzorgen. Bij infecties worden verschillende soorten cytokines geproduceerd waaronder zogenaamde T-helper 1 en T-helper 2 cytokines. T-helper 1 cytokines zijn met name belangrijk voor versterking van de cellulaire immuunrespons, terwijl T-helper 2 cytokines belangrijk zijn voor de humorale immuunrespons. Voorbeelden van T-helper 1 cytokines zijn interferon gamma (IFN-γ), tumour necrosis factor alfa (TNF-α) en interleukine 2 (IL-2). IL-4, IL-5 en IL-13 zijn T-helper 2 cytokines (*Figuur 2*).



Figuur 2. Overzicht van de verschillende soorten afweerreacties.

Ziektebeelden en behandeling

In dit proefschrift worden onderzoeken beschreven die zijn verricht bij mensen met chronische lymfatische leukemie, non-Hodgkin lymfoom (lymfeklierkanker) en multipel myeloom. Leukemie ("bloedkanker") is een kwaadaardige ontaarding van onrijpe (acute leukemie) of rijpere (chronische leukemie) bloedcellen. Chronische lymfatische leukemie is een vorm van bloedkanker waarbij de kwaadaardige cellen rijpe lymfocyten zijn. Bij lymfeklierkanker (ziekte van Hodgkin en non-Hodgkin lymfoom) is sprake van een woekering van kwaadaardige lymfocyten in lymfeklieren en/of beenmerg. Bij multipel myeloom (in Nederland ook bekend als de ziekte van Kahler) is sprake van kwaadaardige plasmacellen (dit zijn rijpe B lymfocyten) in het beenmerg.

De behandeling van deze ziekten bestaat uit chemotherapie, bestraling en/of stamceltransplantatie. Chemotherapie vernietigt vooral cellen die snel delen, maar dit effect beperkt zich helaas niet tot alleen kwaadaardige cellen. Dus naast kankercellen worden bijvoorbeeld ook de, eveneens sneldelende, voorloper bloedcellen in het beenmerg gedood. Wanneer een zeer hoge dosis chemotherapie gegeven moet worden om kankercellen te vernietigen, kan dit permanente schade aan het normale beenmerg aanrichten. De aantasting van de witte bloedcellen door de chemotherapie geeft een verhoogd risico op infecties, terwijl vernietiging van bloedplaatjesproducerende cellen kan resulteren in levensbedreigende bloedingen. Een stamceltransplantatie is dan nodig om aanmaak van nieuwe bloedcellen te waarborgen. Er kunnen 2 verschillende vormen van stamceltransplantatie worden onderscheiden: autoloog (dat wil zeggen met stamcellen van de patiënt zelf) en allogeen (dat wil zeggen met stamcellen van iemand anders, meestal een broer of zus). Voor dit proefschrift zijn de autologe stamceltransplantaties van belang. Hieronder wordt nader ingegaan op het verschil tussen beenmerg- en stamceltransplantaties en hoe een autologe stamceltransplantatie wordt uitgevoerd.

Vroeger werd na hoge dosis chemotherapie beenmergtransplantatie verricht om de bloedaanmaak te waarborgen. Bij patiënten werden eerst met behulp van chemotherapie de zieke cellen uit het beenmerg verdrongen. Vervolgens werd door middel van beenmergpuncties onder narcose beenmerg verzameld

waarna dit werd ingevroren tot het moment van de transplantatie. Na het verzamelen van het beenmerg kreeg de patiënt vervolgens de hoge dosis chemotherapie die gegeven wordt om kankercellen die de eerdere chemotherapie hebben overleefd, alsnog te vernietigen. Vervolgens wordt het beenmerg teruggegeven (de beenmergtransplantatie). Een nadeel van deze procedure is dat het verzamelen van voldoende beenmerg erg pijnlijk is en dus onder algehele narcose verricht moet worden. Tegenwoordig wordt vrijwel alleen nog maar gebruik gemaakt van stamcellen als bron voor een autologe transplantatie. Hierbij wordt ook eerst chemotherapie gegeven om de kwaadaardige cellen uit het beenmerg te elimineren. Vervolgens krijgen de patiënten onderhuidse injecties met een groeifactor (ook een soort cytokine) waardoor de stamcellen, die normaliter alleen in het beenmerg zitten, zich sterk gaan delen en ook (gedurende een beperkte periode) in het bloed terecht komen. Wanneer voldoende stamcellen in het bloed aanwezig zijn, wordt de patiënt aan een leukaferese (een soort dialyseapparaat)-apparaat gekoppeld. Via een infuus in de ene arm (of in de lies) wordt het bloed uit het lichaam gehaald en worden door het apparaat de stamcellen eruit gefilterd, waarna door het tweede infuus in de andere arm het resterende bloed wordt terug gegeven. Na het verzamelen van de stamcellen wordt de hoge dosis chemotherapie gegeven, gevolgd door teruggave van de stamcellen aan de patiënt.

Patiënten die een autologe stamceltransplantatie hebben ondergaan, hebben gedurende lange tijd (maanden tot jaren) een gestoorde afweer. De stamcellen moeten immers weer gaan uitrijpen tot afweercellen waarna het nog een tijd duurt voordat de afweercellen weer volledig functioneel zijn. Bij patiënten met CLL is de afweer om heel andere redenen gestoord. Zo maken de kwaadaardige B-lymfocyten geen afweerstoffen en er blijven nog maar weinig functionele normale B-lymfocyten over. Daarbovenop veroorzaakt een eventuele behandeling met chemotherapie een verdere afname van de afweerfuncties. Het onderzoek beschreven in dit proefschrift is gericht op preventie van infecties in deze patiëntengroepen en op analyse van herstel van afweer na autologe stamceltransplantatie.

Samenvatting van dit proefschrift

In dit proefschrift worden, na een inleidend hoofdstuk (**Hoofdstuk 1**), de effecten van vaccinaties bij patiënten met chronische lymfatische leukemie beschreven. Tevens worden studies naar het effect van vaccinaties en het herstel van afweer bij patiënten die een autologe stamceltransplantatie hebben ondergaan, gepresenteerd.

Patiënten met chronische lymfatische leukemie
Chronische lymfatische leukemie (CLL) is een ziekte die voornamelijk bij ouderen voorkomt (meer dan 70% van de patiënten is ouder dan 60 jaar op het moment dat de diagnose gesteld wordt). CLL kan worden ingedeeld in verschillende stadia, bijvoorbeeld volgens de Rai-classificatie (zie *Tabel* 1). Afhankelijk van het ziektestadium varieert de overleving van nog geen 2 jaar tot meer dan 15 jaar.

Tabel 1. Indeling van CLL volgens Rai.

Stadium 0	verhoogd aantal lymfocyten in het bloed (meer dan 15 miljard per liter bloed)
Stadium 1	gelijk aan Stadium 0 met daarbij vergrote lymfklieren
Stadium 2	gelijk aan Stadium 0 met daarbij vergrote lever en/of milt, eventueel vergrote lymfklieren
Stadium 3	gelijk aan Stadium 0 met daarbij bloedarmoede, eventueel vergrote lymfklieren, eventueel vergrote lever en/of milt
Stadium 4	gelijk aan Stadium 0 met daarbij een verlaagd aantal bloedplaatjes (minder dan 100 miljard per liter bloed), eventueel vergrote lymfklieren, eventueel vergrote lever en/of milt

Patiënten met CLL zijn verhoogd gevoelig voor infecties ten gevolge van de ziekte zelf (de afwijkende B-lymfocyten maken geen afweerstoffen en normale B-lymfocyten zijn verlaagd in aantal) en door de chemotherapie. Omdat 50-60% van de CLL-patiënten uiteindelijk overlijdt aan een infectie, is het erg belangrijk om infecties zoveel mogelijk te voorkomen. Een manier om dit te bewerkstelligen is het nog resterende afweersysteem maximaal te benutten en te versterken door vaccinatie, bijvoorbeeld met het influenza virus vaccin (de jaarlijkse “griepprik”). Door een vaccinatie wordt een afweerreactie tegen een bacterie of virus

opgewekt met als resultaat de vorming van grote hoeveelheden geheugencellen. Wanneer de patiënt daarna besmet raakt door dezelfde bacterie of hetzelfde virus, zullen de opgebouwde geheugencellen in korte tijd veel afweerstoffen produceren waardoor de infectie effectief bestreden wordt. Helaas maken patiënten met CLL onvoldoende hoeveelheden afweerstoffen aan na een griepvaccinatie en zijn dus niet goed beschermd. In **Hoofdstuk 2** wordt het effect van een extra vaccinatie (zogenaamde booster vaccinatie) met het influenza virus vaccin, 3 weken na de eerste dosis, beschreven bij patiënten met CLL. Na de eerste vaccinatie was het aantal responders (dit zijn patiënten met een voldoende productie van afweerstoffen) slechts 10%. De boostervaccinatie zorgde voor een toename van het aantal responders van 10% naar ongeveer 25%. Hoewel dit een verbetering is, is dit nog steeds een lage respons vergeleken met gezonde jonge volwassenen en ouderen. Toch is het raadzaam, mede gezien het vrijwel ontbreken van bijwerkingen, patiënten met CLL jaarlijks te vaccineren met het influenza virus vaccin. Bij 10-25% van de patiënten voorkom je daarmee infecties met het griepvirus of hieraan gerelateerde complicaties, terwijl er voor de overige patiënten geen grote nadelen zijn (mogelijke bijwerkingen zijn roodheid en pijn ter plaatse van de injectieplaats).

De bacteriële infecties bij CLL-patiënten worden vaak veroorzaakt door *Haemophilus influenzae* type b (*Hib*) en *Streptococcus pneumoniae* (pneumokok). Deze bacteriën kunnen longontsteking, middenoorontsteking en hersenvliesontsteking veroorzaken. Mensen met een verminderde afweerfunctie, zoals jonge kinderen, chronisch zieken en ouderen, hebben een verhoogde vatbaarheid voor deze infecties. In Nederland worden kinderen die het Rijksvaccinatieprogramma volgen, gevaccineerd met het *Hib*-vaccin en sinds april 2006 ook met een pneumokokkenvaccin. In de Verenigde Staten worden vaccinaties met *Hib*- en pneumokokkenvaccin ook routinematig aangeboden aan ouderen vanaf 65 jaar. De patiënten met CLL zou je dus eigenlijk om 2 redenen met deze vaccins willen vaccineren: vanwege hun leeftijd en vanwege hun verminderde afweer ten gevolge van hun ziekte. De respons van het immuunsysteem op *Hib*- en pneumokokkenvaccins bij patiënten met CLL is echter vaak suboptimaal. Patiënten die zich nog in een vroeg ziektestadium bevinden, hebben over het algemeen een betere respons dan patiënten met een vergevorderd ziektestadium. Op verschillende manieren wordt geprobeerd om deze respons op vaccinaties te verbeteren. Ranitidine (Zantac®) is een middel dat de maagzuurproductie remt en waarvan bekend is dat het effecten

op de afweer heeft. In **Hoofdstuk 3** wordt beschreven hoe patiënten met CLL gevaccineerd werden met *Hib*- en pneumokokkenvaccin waarbij aan de helft van de onderzochte patiënten ranitidine werd gegeven. De patiënten die ranitidine kregen, lieten een betere respons zien op *Hib*-vaccinatie dan de groep patiënten die geen ranitidine kreeg. De respons op pneumokokkenvaccinatie was vergelijkbaar in beide groepen. Hoewel ranitidine dus positieve effecten op de respons op *Hib*-vaccinatie heeft, zijn er recent ook nadelen beschreven. Er zijn aanwijzingen dat tijdens behandeling met ranitidine een grotere kans op het ontwikkelen van longontsteking bestaat. Daarom adviseren wij het voorschrijven van ranitidine tijdens vaccinatie in patiënten met CLL niet. Aangezien CLL-patiënten vroeg in de ziekte in het algemeen een betere respons op vaccinatie laten zien, is het verstandiger om in een zo vroeg mogelijk ziektestadium te vaccineren met een *Hib*- en pneumokokkenvaccin.

Patiënten die een autologe stamceltransplantatie hebben ondergaan
Patiënten, die een autologe stamceltransplantatie (aSCT) ondergaan, hebben gedurende lange tijd na transplantatie een gestoorde afweer. De hoge dosis chemotherapie vernietigt (vrijwel) alle bloedcellen, dus ook de geheugencellen die belangrijk zijn voor de afweer. Daarom wordt in alle richtlijnen betreffende stamceltransplantaties geadviseerd aSCT-patiënten opnieuw te vaccineren met onder andere difterie, tetanus en polio vaccin (DTP), *Hib* en pneumokokkenvaccin.

De meest gebruikte vaccinatieschema's starten 12 maanden na transplantatie. Te vroeg vaccineren na transplantatie is vaak weinig effectief en kan mogelijk lijden tot "tolerantie". Een vaccinatie zeer kort na transplantatie resulteert in geen of weinig afweerstoffen omdat er nog te weinig (rijpe) afweercellen zijn. Wanneer na vaccinatie een infectie optreedt met dezelfde bacterie of virus, dan herkennen de afweercellen de bacterie of het virus niet als vreemd en vindt geen afweerreactie plaats: er is tolerantie ontwikkeld. Anderzijds geldt dat hoe later gevaccineerd wordt, hoe langer de patiënten een onnodig verhoogd infectierisico lopen. Van 2002-2006 werd de VAST-studie (Vaccination after Autologous Stem cell Transplantation) uitgevoerd waarbij op 6 maanden na transplantatie werd gestart met het vaccinatieschema. Het doel van dit onderzoek was het effect van een dergelijk vroeg vaccinatieschema na aSCT te evalueren. Aan dit onderzoek deden patiënten

uit het St. Antonius Ziekenhuis te Nieuwegein, Medisch Spectrum Twente te Enschede en Meander Medisch Centrum te Amersfoort mee. Het onderzoek werd gecoördineerd vanuit het St. Antonius Ziekenhuis. Autologe SCT-patiënten (met als onderliggende ziekte voornamelijk non-Hodgkin lymfoom of multipel myeloom) werden op 6, 8 en 14 maanden na transplantatie gevaccineerd met DTP, *Hib* en pneumokokkenvaccin. Dit schema leverde een goede respons op, zowel kwantitatief (de hoeveelheid afweerstoffen die werden geproduceerd) als kwalitatief (de functionele capaciteit van de afweerstoffen), zoals beschreven is in **Hoofdstuk 4**. Ernstige bijwerkingen traden niet op. Het bleek dus veilig en effectief wat betreft het opwekken van afweerstoffen om bij aSCT-patiënten reeds 6 maanden na transplantatie te starten met revaccinaties. Uiteraard is het uiteindelijke doel van vaccineren het voorkómen van infecties en is de hoeveelheid afweerstoffen slechts een afgeleide daarvan. Het aantal patiënten in de VAST-studie was echter te klein om de effectiviteit van dit vaccinatieschema ten aanzien van de reductie van het aantal infecties te toetsen.

In de VAST-studie werd niet alleen gekeken naar de specifieke respons op de vaccinatie, maar ook meer algemeen naar het herstel van de verschillende witte bloedcellen (**Hoofdstuk 5**). Zoals eerder uitgelegd, zijn onder andere T- en B-lymfocyten nodig om infecties te bestrijden. Het totale aantal T-lymfocyten bleek tijdens de hele onderzoeksperiode (vanaf 3 tot 14 maanden na transplantatie) normaal te zijn. De onderverdeling van de T-cellen bleek wel anders te zijn: het aantal helper T-cellen was blijvend verlaagd ten opzichte van het aantal cytotoxische T-cellen (van belang voor de cellulaire afweer). Het is dus opvallend dat ondanks lage aantallen T-helper cellen, die belangrijk zijn voor het in gang zetten van productie van afweerstoffen door B-cellen, de respons op vaccinaties goed was. Het gemiddeld aantal B-lymfocyten was 3 maanden na transplantatie laagnormaal en was volledig genormaliseerd 14 maanden na transplantatie. Patiënten met een te laag aantal B-lymfocyten hadden meerdere *Hib*-vaccinaties nodig om een voldoende hoeveelheid afweerstoffen te produceren, terwijl patiënten met voldoende B-lymfocyten na één *Hib*-vaccinatie voldoende afweerstoffen produceerden. Een voldoende aantal B-lymfocyten is dus wel een vereiste om een goede vaccinatierespons te verkrijgen.

Vervolgens werd in het kader van de VAST-studie ook de cytokineproductie geanalyseerd (**Hoofdstuk 6**). Cytokines zijn eiwitten die de communicatie tussen ver-

schillende cellen tijdens afweerreacties verzorgen (zie *Figuur 2*). Bij onvoldoende cytokineproductie zal de productie van afweerstoffen door B-lymfocyten niet goed op gang komen en zullen de cellulaire afweerreacties door cytotoxische T-lymfocyten ook minder zijn. Hoewel van 3 tot 14 maanden na stamceltransplantatie een toename werd gezien van alle gemeten cytokines, bleek dat de productie van T-helper 2 cytokines beter was dan van T-helper 1 cytokines. Met andere woorden, de cytokines die belangrijk zijn voor de productie van afweerstoffen herstellen zich beter en eerder dan de cytokines die belangrijk zijn voor de cellulaire afweer. Deze resultaten sluiten goed aan bij de bevindingen betreffende de respons op vaccinaties; die is relatief goed 6 maanden na transplantatie en neemt verder toe gedurende de periode daarna. De verschillende onderdelen van de humorale immuunrespons (T-helper cellen, T-helper 2 cytokines, B-lymfocyten en afweerstoffen) zijn vooral belangrijk voor de afweer tegen bacteriën. De cellulaire immuunrespons (met onder andere cytotoxische T-cellen en T-helper 1 cytokines) is belangrijk voor de afweer tegen virussen. Bij patiënten die een autologe stamceltransplantatie hebben ondergaan worden relatief veel virale infecties gezien, met name in de zogenaamde late periode (dat wil zeggen meer dan 3 maanden na transplantatie). Vooral infecties met het varicella zoster virus (het virus dat waterpokken en gordelroos veroorzaakt) komen frequent voor. Een voor de hand liggende verklaring hiervoor is de bevinding dat de productie van T-helper 1 cytokines gedurende het eerste jaar na transplantatie achterblijft.

Tenslotte bevat **Hoofdstuk 7** een samenvatting en algemene discussie van de behaalde resultaten. Concluderend blijkt uit dit proefschrift dat de griepprik slechts beperkte waarde heeft en dat vaccinaties met *Hib*- en pneumokokkenvaccin een suboptimale immuunrespons geven bij patiënten met CLL. Het toedienen van ranitidine heeft een positief effect op de respons op *Hib*-vaccinatie maar niet op pneumokokkenvaccinatie. Met de resultaten van de VAST-studie is meer inzicht verkregen in de respons op vaccinaties en herstel van immuniteit na autologe stamceltransplantatie.

Aanbevelingen voor toekomstig onderzoek

Omdat vaccinatie met de huidige beschikbare vaccins en volgens de door ons gehanteerde schema's lang niet altijd een voldoende respons oplevert, is nog veel vervolgonderzoek nodig om afdoende infectiepreventie bij patiënten met een kwaadaardige hematologische aandoening te bewerkstelligen. Enkele voorbeelden zijn:

» Voor patiënten met CLL is een analyse van de werkzaamheid van nieuwe griepremmers en een kosten-baten analyse van het preventief geven van antibiotica van belang.
» Ten aanzien van vaccinaties na aSCT is het nog onbekend of langere tijd na transplantatie (bijvoorbeeld na 5 jaar zoals gebruikelijk is bij patiënten van wie de milt verwijderd is) nogmaals gevaccineerd moet worden met pneumokokken- (en eventueel ook *Hib*-) vaccin.
» Gezien het veelvuldig optreden van infecties met varicella zoster virus na aSCT kan het recent ontwikkelde varicella zoster virus vaccine van belang zijn voor aSCT-patiënten. De effectiviteit en veiligheid van dit vaccin na aSCT zijn echter nog niet volledig duidelijk. Het varicella zoster vaccin is namelijk een verzwakt levend vaccin, dat wil zeggen dat het virus in zijn geheel (maar grotendeels geïnactiveerd) wordt toegediend. Dit in tegenstelling tot de *Hib*- en pneumokokkenvaccins, waarbij gevaccineerd wordt met een onderdeel van de bacteriewand. Een nadeel van verzwakt levende vaccins is dat de patiënt een (meestal lichte) infectie kan krijgen door de vaccinatie zelf. Bij mensen met een verminderde afweer, zoals patiënten die een aSCT hebben ondergaan, kan zo'n infectie gevaarlijk zijn.
» In de afgelopen jaren zijn nieuwe geneesmiddelen ontwikkeld zoals rituximab (voor patiënten met non-Hodgkin lymfoom), bortezomib en thalidomide (voor patiënten met de ziekte van Kahler). De effecten van deze geneesmiddelen op afweer en vaccinatierespons moeten nog verder in kaart worden gebracht.
» Tenslotte vinden sinds enkele jaren allogene stamceltransplantaties plaats waarbij een minder schadelijke vorm van chemotherapie en/of bestraling wordt gegeven ten opzichte van de klassieke allogene stamceltransplantaties ("reduced-intensity" stamceltransplantatie, RIST). Veel patiënten met de ziekte van Kahler ondergaan tegenwoordig eerst een autologe stamceltransplantatie, gevolgd door een allogene RIST. Onderzoek naar de effecten van een dergelijk intensief behandelschema op immuniteit en vaccinatierespons is van groot belang. In het VU Medisch Centrum is reeds gestart met onderzoek naar de respons op vaccinaties bij patiënten die een allogene RIST hebben ondergaan.

Dankwoord

Het heeft ruim 7 jaar geduurd tot dit proefschrift voltooid was. Velen ben ik dank verschuldigd. Sommigen vanwege een directe bijdrage aan dit boekje, vele anderen hebben echter indirect geholpen.

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Douwe Biesma, co-promotor. Mede door jouw enthousiaste en vooral voortvarende begeleiding is het me gelukt om tijdens mijn opleiding dit promotieonderzoek te doen. Ik heb meerdere malen na het rondsturen van een nieuw artikel ten onrechte gedacht: “Zo, nu even een weekend vrij!”. Meestal had ik echter binnen 24 uur een gecorrigeerde versie van je terug. Ik heb nog nooit een man gezien die zo efficiënt werkt!

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Roel. "*In een boerengemeenschap is het niet vreemd iemand met een stok of een staf te zien lopen. Die heb je altijd bij je. Hij helpt je om de pas erin te houden. Hij is een kruk om op te steunen en helpt je om niet te wankelen. [...] Je voelt je veilig met hem.*"
Deze woorden van Nelson Mandela als postuum eerbetoon aan zijn vriend Walter Sisulu, geven perfect weer wat jij voor mij betekend hebt tijdens het tot stand komen van dit proefschrift. Slechts één ding ontbreekt: relativeringsvermogen. Zullen we maar zeggen: "Op naar de volgende 17 jaar"?

Curriculum vitae

Ankie van der Velden werd geboren op 26 november 1973 te Venlo. In 1991 behaalde zij haar VWO-diploma aan het Collegium Marianum te Venlo waarna zij startte met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Na het behalen van het artsendiploma in december 1998 werkte zij gedurende ruim een jaar als AGNIO interne geneeskunde in het St. Antonius Ziekenhuis te Nieuwegein. Gedurende dit jaar werd gestart met het eerste deel van het onderzoek dat beschreven is in dit proefschrift. Van mei 2000 tot januari 2001 volgde zij het eerste deel van van de opleiding tot internist in het Universitair Medisch Centrum Utrecht (opleider: Prof. Dr. D.W. Erkelens), waarna zij de opleiding vervolgde in het St. Antonius Ziekenhuis te Nieuwegein (opleider: Dr. H.C.M. Haanen). Van januari 2004 tot januari 2005 verrichtte zij in het kader van een KWF-beurs een groot deel van het onderzoek dat beschreven is in dit proefschrift. Vanaf januari 2005 vervolgde zij haar opleiding aan het VU Medisch Centrum te Amsterdam (opleider: Prof. Dr. S.A. Danner) met als aandachtsgebied hematologie (opleider: Prof. Dr. P.H. Huijgens). Zij werd geregistreerd als internist op 1 oktober 2006 en als hematoloog op 1 mei 2007. Thans is zij werkzaam als fellow oncologie in het VU Medisch Centrum (opleider: Prof. Dr. E. Boven).

List of abbreviations

AI	avidity index
aSCT	autologous stem cell transplantation
CAP	community acquired pneumonia
CLL	chronic lymphocytic leukaemia
Cpm	counts per minute
CPS	common cell wall polysaccharide
DTP	diphteria, tetanus and polio
ELISA	enzyme-linked immunosorbent assay
Hib	Haemophilus influenzae type B
Ig	immunoglobulin
IL	interleukin
IPD	invasive pneumococcal disease
IPI	invasive pneumococcal infection
IVIG	intravenous immunoglobulin
MM	multiple myeloma
NaSCN	sodium thiocyanate
NHL	non-Hodgkin's lymphoma
NK cells	natural killer cells
PHA	phytohaemagglutinin
Th1	type 1 T-helper cell
Th2	type 2 T-helper cell
TT	tetanus toxoid
VAST	Vaccination after Autologous Stem cell Transplantation trial
VZV	varicella zoster virus